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THE UNIVERSITY OF ALBERTA

A STUDY OF PEPTIDOGLYCAN BIOSYNTHESIS IN AN *IN VITRO* SYSTEM FROM *MICROCOCCUS SODONENSIS*

BY

©

Susan E. Jensen

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
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DEPARTMENT OF MICROBIOLOGY

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SPRING, 1975

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "A study of Peptidoglycan Biosynthesis in an *In Vitro* System from *Micrococcus sodonensis*" submitted by Susan E. Jensen in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

Membrane suspensions were prepared from stationary-phase cells of Micrococcus sodonensis and were capable of carrying out transglycosidation when supplied with UDP-MurNAc-pentapeptide, UDP-GlcNAc, and appropriate cofactors. The peptidoglycan produced in these assay systems could be completely digested by lysozyme, but was uncrosslinked as determined by its insensitivity to cephaloridine and the absence of release of free alanine. The peptidoglycan product was non-homogeneous and consisted of 76 percent "soluble" and 24 percent "insoluble" material. "Soluble" peptidoglycan was primarily of small molecular weight although some large molecular weight material was present. "Insoluble" peptidoglycan was found to be "insoluble" by virtue of its non-covalent attachment to membrane fragments, since it could be solubilized by butanol extraction. This material consisted of approximately equal amounts of large and small molecular weight species. Molecular weight determinations on "soluble" *in vitro* synthesized peptidoglycan indicated that the small molecular weight material (20,000 daltons) consisted of uniform linear glycan strands made up of about 20 disaccharide-peptide units, while the large molecular weight material (70,000-130,000 daltons) was a mixture of glycan strands ranging from 74 to greater than 130 disaccharide-peptide units in length.

Stationary-phase membrane suspensions also contained a pyrophosphatase activity which was expressed only when UDP-GlcNAc was omitted from the transglycosidase reaction mixture. This enzyme degraded the elevated levels of lipid linked-P-P-MurNAc-pentapeptide which accumulated

when peptidoglycan biosynthesis was interrupted. The products of this enzyme are MurNAc-pentapeptide and UMP.

Neither transglycosidase nor pyrophosphatase activities could be solubilized except to a limited degree.

Membrane suspensions prepared from exponential-phase cells of *M. sodonensis* contained not only transglycosidase and pyrophosphatase activities, but also a third enzymatic activity, an N-acetylmuramyl-L alanine amidase which hydrolysed the substituent pentapeptide from *in vitro* synthesized peptidoglycan but would not degrade peptidoglycan precursors (UDP-MurNAc-pentapeptide or MurNAc-pentapeptide) or products of lysozyme digestion of peptidoglycan (di-and tetrasaccharide-peptides). It would degrade both large and small molecular weight fractions of *in vitro* synthesized peptidoglycan as well as *in vivo* synthesized peptidoglycan contained in purified cell wall preparations.

The amidase activity was also demonstrable as present in large amounts in the cell wall fraction of exponential-phase cells but in spite of this, the enzyme did not appear to be autolytic in the intact cell. Amidase activity could be readily solubilized from both the cell membrane and cell wall preparations by LiCl extraction. Gel filtration analyses of cell wall and cell membrane derived amidases indicated both to have a molecular weight greater than 150,000 daltons.

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ABBREVIATIONS

DEAE	- diethylaminoethyl
DNP	- dinitrophenyl
EDTA	- ethylenediaminetetraacetate
FDNB	- fluorodinitrobenzene
Kav	- distribution coefficient between liquid phase and gel phase
MIC	- minimum growth inhibitory concentration
NADH	- reduced nicotinamide-adenine dinucleotide
TCA	- trichloroacetic acid
TCS	- trypticase soy medium
Tris	- tris(hydroxymethyl)aminomethane
TME	- Tris-mercaptoethanol buffer
UDP-glucose	- uridine diphospho glucose
UDP-GlcNAc	- uridine diphospho N-acetyl-D-glucosamine
UDP-MurNAc-pentapeptide	- uridine diphospho N-acetyl-muramyl-L alanyl-D glutamyl-L lysyl-D alanyl-D alanine
Ve	- elution volume
Vo	- void volume
Vt	- total volume

INTRODUCTION

The chemical composition of bacterial cell walls together with the biosynthetic mechanisms responsible for their synthesis have been undergoing intensive investigation for the past twenty years. Cell walls are the essential structural components of bacterial cells which function in the maintenence of cellular integrity in hypotonic environments. Peptidoglycan is the component of the cell wall which imparts strength and rigidity to the structure. Cell walls of Gram-positive organisms have very thick peptidoglycan layers comprising 50 to 90 percent of the dry weight of the wall and are capable of containing intracellular osmotic pressures as high as 25 atmospheres in some cases (Mitchell and Moyle, 1956). Cell walls of Gram-negative organisms characteristically have less peptidoglycan (5 to 15 percent of the dry weight of the wall) and are correspondingly weaker than their Gram-positive counterparts, sustaining maximum intracellular osmotic pressures of only 5 to 6 atmospheres (Mandelstam, 1962).

Non-peptidoglycan components of Gram-positive cell walls typically consist of techoic acids and polysaccharides as well as small amounts of protein and lipid. The exact organization of these non-peptidoglycan components within the cell wall is not known. The Gram-positive cell wall appears homogeneous and undifferentiated upon electron microscopic examination. In some organisms, techoic acids are known to be synthesized in close proximity to peptidoglycan and the two polymers are covalently crosslinked at the time of synthesis (Glaser, 1973). These techoic acids would be interspersed throughout the cell wall. Some polysaccharides are also covalently attached to the peptidoglycan, but in other instances where covalent attachment does not exist the polysaccharides are still integrated throughout the cell wall and can not be removed except by enzymatic degradation of the peptidoglycan network (Johnson, 1971). Both techoic acids and polysaccharides frequently contribute to the antigenicity of the bacterial cell and as such they must exist at and may possibly predominate at the external surface of the cell wall (Johnson, 1971; Braatz and Heath, 1974). Gram-negative cell walls are more complex than Gram-positive cell walls, giving a layered appearance upon electron microscopic observation. In addition to the thin band of peptidoglycan which lies adjacent to the cell membrane, the walls also contain high levels of lipid in the form of lipoprotein and lipopolysaccharide. The outer double track layer, characteristic of Gram-negative cell walls, is a membrane-like structure composed of lipopolysaccharide. This outer double track layer provides some structural support for the cell, but it also forms a partial permeability barrier which results in the decreased sensitivity of these cells to many antibiotics and peptidoglycan degrading enzymes (Tseng and Bryan, 1974).

Since the cell wall is a necessary structural component for all bacteria with the exception of Mycoplasma, L-forms, and some halophiles, and because there is no eucaryotic system which contains peptidoglycan, antibiotics which prevent cell wall formation by interrupting peptidoglycan synthesis have become valuable chemotherapeutic agents. Elucidating the mechanisms of action of these antibiotics first required a full understanding of the sequence of reactions involved in normal peptidoglycan biosynthesis. One approach to this problem involved the isolation and chemical characterization of cell walls from a wide range of organisms.

This first became possible in 1951 when Salton and Horne described a technique for isolating bacterial cell walls (Salton and Horne, 1951). After extensive purification to remove contaminating cellular material the peptidoglycan composition of the cell wall preparation was determined using a variety of specific chemical assays. Arrangement of the constituent hexosamine and amino acid residues was then determined either by partial acid hydrolysis, or by enzymatic degradation using cell wall lytic enzymes of known specificities (Schleifer and Kandler, 1967; Ghuysen, 1968). Cell wall structure has been so widely studied by these techniques that it is now proposed as a taxonomic criterion for classifying and identifying unknown microorganisms (Schleifer and Kandler, 1972). Possible sources of error in such an approach have been pointed out by the findings that while peptidoglycan structures do appear to be species specific, they can change both in chemical composition and in physical arrangement when exposed to changes in the growth medium (Johnson and Campbell, 1972; Hammes _et al._, 1973; Hilderman and Riggs, 1973).

The elucidation of peptidoglycan structures for a range of microorganisms indicated that certain aspects of the structure are universal while other characteristics differ markedly from one species to another. All peptidoglycan types consisted of a linear glycan backbone made up of repeating sequences of N-acetylmuramic acid and N-acetylglucosamine residues. The average length of these glycan strands differed from one organism to another but lengths in the range of 6 to 12 disaccharide units were typically found (Ward, 1973; Higgins and Shockman, 1971). The muramic acid residues of glycan chains carried short substituent peptides containing 4 or 5 amino acids of both D and L configurations. Adjacent peptidoglycan strands were joined together by means of these substituent peptides. The linkage could be direct or it could involve the intervention of a crossbridging peptide which in turn joined the two substituent peptides. It was the amino acid composition of these substituent and crossbridging peptides which gave rise to inter-species differences (Ghuysen, 1968; Petit _et al._, 1966; Munoz _et al._, 1966). The uniformity of the basic peptidoglycan structure suggested that a common mechanism of formation might exist for all bacterial species with minor variations resulting in different peptide compositions and modes of crossbridging.

The study of the biosynthesis of peptidoglycan was initiated by the work of Park who discovered the presence of an intracellular complex uridine nucleotide containing N-acetylmuramic acid and five amino acids (Park, 1952a; 1952b; 1952c). This compound, UDP-N-acetylmuramic acid-pentapeptide (UDP-MurNAc-pentapeptide) accumulated in _Staphylococcus aureus_ when exposed to growth inhibitory concentrations of penicillin. The amino acids formed a pentapeptide with a sequence of L alanyl-D isoglutamyl-L lysyl-D alanyl-D alanine, which was joined to the N-acetylmuramic acid residue by a muramyl-L alanine amide linkage. The accumulation of this compound was correctly assumed to be related to the inhibition of peptidoglycan biosynthesis since the sugar-peptide portion of the nucleotide so closely resembled a portion of the peptidoglycan structure. On this basis, peptidoglycan biosynthesis was postulated to be analogous to extracellular polysaccharide biosynthesis which takes place by transfer of monosaccharides from nucleotide diphosphate donor molecules (Leloir, 1965). Peptidoglycan biosynthesis appeared to involve the assembly of preformed sugar-peptide subunits which were synthesized intracellularly.

The synthetic steps leading up to the formation of UDP-MurNAc-pentapeptide are mediated by soluble, intracellular enzymes, and as such they were amenable to conventional methods investigation. D cycloserine, an antibiotic which interrupts the synthesis of UDP-MurNAc-pentapeptide, made possible the individual study of some of the synthetic steps involved. D cycloserine is believed to competitively inhibit alanine racemase, the enzyme needed to produce D alanine, and D alanyl-D alanine synthetase, the enzyme which forms the C terminal dipeptide. Both enzymes are affected presumably because of a conformational similarity between D cycloserine and the normal substrates (Strominger _et al._, 1959; 1967). UDP-MurNAc-pentapeptide was first successfully synthesized _in vitro_ using crude enzyme extracts from _S. aureus_ and more recently from _Escherichia coli_ and _Bacillus subtilis_ (Ito and Strominger, 1964; Ito _et al._, 1966; Lugtenberg, 1972; Egan _et al._, 1973). The peptide portion of the precursor prepared from the latter two organisms has diaminopimelic acid replacing lysine, a difference reflected in the peptidoglycan structures of the organisms.

Alternative _in vivo_ methods for producing UDP-MurNAc-pentapeptide include the exposure of cells to growth inhibitory concentrations of vancomycin. Vancomycin, like penicillin, prevents peptidoglycan biosynthesis, but vancomycin inhibits at an earlier step in the synthetic sequence and as a result UDP-MurNAc-pentapeptide can be accumulated in a wide range of species, some of which do not respond to penicillin (Lugtenberg _et al._, 1971). The most successful method of promoting intracellular accumulation of UDP-MurNAc-pentapeptide relies on the deprivation of divalent cations by means of a chelating agent. All of the membrane bound and extracellular steps of peptidoglycan biosynthesis have an absolute requirement for

Mg^{2+} and can not proceed in its absence. Since this method is not dependent on the sensitivity of a given organism to an antibiotic, it is effective for an even wider range of species (Garrett, 1969).

Examining the conversion of UDP-MurNAc-pentapeptide to peptidoglycan was a much more complex problem than examining its synthesis. It involved following the passage of this precursor through the membrane and its assembly and organization into a complex insoluble structure. The enzymes mediating these reactions are particulate and not exposed to normal intracellular control and regulatory mechanisms. Detailed analysis of these later steps of peptidoglycan biosynthesis became possible with the development of an _in vitro_ assay system from _S. aureus_ capable of synthesizing peptidoglycan (Meadow _et al._, 1964; Chatterjee and Park, 1964). UDP-MurNAc-pentapeptide was an essential substrate for this _in vitro_ system but synthesis of peptidoglycan was also dependent on the simultaneous addition of UDP-N-acetylglucosamine (UDP-GlcNAc). Controlled production of the UDP-MurNAc-pentapeptide precursor allowed incorporation of specific, radioactively labelled components, making the _in vitro_ assay systems more sensitive and convenient to use. In each case the UDP-MurNAc-pentapeptide was prepared from the same or a related organism used to produce the particulate enzymes. This ensured that the substituent peptide composition of the nucleotide precursor corresponded with the specificity of the enzymes involved. Modified peptide sequences which do not occur naturally have been prepared abiotically by solid phase synthesis (Nieto and Perkins, 1971). These synthetic compounds can serve as substrates or inhibitors in some _in vitro_ assay systems.

As well as the appropriate substrates, _in vitro_ assay systems also required a source of the particulate enzymes involved in peptidoglycan biosynthesis. A suspension of isolated membrane fragments has most commonly been used but the peptidoglycan synthesizing ability of these membrane fragments was found to be strongly influenced by the method of cell breakage used in preparation of the membranes. Grinding whole cells with alumina in a mortar and pestle was successfully used to prepare membrane suspensions capable of synthesizing peptidoglycan from *S. aureus* and *Micrococcus lysodeikticus* (Anderson *et al.*, 1965; 1967). When membranes were prepared from cells broken by ultrasonic vibration or by shaking with glass beads, the resulting membrane fragments could not synthesize peptidoglycan unless the *in vitro* assay system was supplemented with pieces of filter paper (Chatterjee and Park, 1964; Anderson *et al.*, 1965). The paper fibers provided an insoluble matrix which helped organize the particulate enzymes into an active arrangement. Grinding or shaking whole cells with plastic beads also resulted in cell breakage but the membrane fragments were able to synthesize peptidoglycan without the filter paper support (Lugtenberg *et al.*, 1971).

Peptidoglycan synthesized by a membrane based *in vitro* system from *S. aureus* is a linear, uncrosslinked glycan polymer bearing substituent peptides which retain all of the amino acids present in the nucleotide precursor. Incorporation of GlcNAc and MurNAc-pentapeptide into peptidoglycan also results in the release of equimolar amounts of UDP, UMP, and inorganic phosphate (Park and Chatterjee, 1966). The first recognizable step of *in vitro* peptidoglycan biosynthesis involves the transfer of phospho-MurNAc-pentapeptide from the nucleotide precursor to a membrane bound phospholipid which serves as a carrier for transporting disaccharide-pentapeptide subunits across the membrane. UMP is released at this stage (Anderson et al., 1965; 1967). This carrier lipid was identified as a C_{55} isoprenoid alcohol, undecaprenol, which is active in the monophosphate form (Higashi et al., 1967). The phospho-MurNAc-pentapeptide becomes attached to the phosphate residue in the carrier lipid forming a pyrophosphate bond. Omitting UDP-GlcNAc from the in vitro assay system completely prevents peptidoglycan biosynthesis but formation of the MurNAc-pentapeptide bound carrier lipid intermediate can still take place. Bacitracin is an antibiotic which interrupts peptidoglycan biosynthesis by preventing the formation of carrier lipid linked precursors. Bacitracin and Ca^{2+} together form a complex with C_{55} isoprenyl pyrophosphate, the inactive form of the carrier lipid. The phosphatase which normally regenerates C_{55} isoprenyl pyrophosphate back to the active monophosphate form cannot attack the complex (Stone and Strominger, 1971; 1972; Schechter et al., 1972). Since carrier lipids are present in limited amounts and require regeneration after each cycle of peptidoglycan biosynthesis, bacitracin rapidly depletes the supply of active carrier lipid molecules.

The second step of peptidoglycan biosynthesis involves the transfer of GlcNAc to the carrier lipid bound MurNAc-pentapeptide with the release of UDP. A β-1, 4-glycosidic linkage joins the GlcNAc residue to the MurNAc residue to form the completed disaccharide-pentapeptide subunit which is still bound to the carrier lipid by the original pyrophosphate bond. GlcNAc cannot be transferred from UDP-GlcNAc to the carrier lipid without the prior attachment of MurNAc-pentapeptide. Modification of the substituent peptide portion of the disaccharide-pentapeptide-P-P-phospholipid intermediates take place at this stage in vivo. These modifications can also be produced in vitro by making additions to the reaction mixtures. In *M. lysodeikticus*, the substituent peptide is modified by the addition of a glycine residue to the free α carboxyl group of glutamic acid in a reaction requiring ATP (Katz *et al.*, 1967). The formation of crossbridging peptides can also take place at this stage. *S. aureus* has a crossbridging peptide consisting of five glycine residues attached to the free ε amino group of lysine. These glycine residues are incorporated by a mechanism totally different from the ATP dependent mechanism of *M. lysodeikticus*. In *S. aureus*, glycine is activated as glycyl-tRNA and then transferred to the intermediate without the participation of ATP. The glycine specific tRNA involved is distinct from the tRNA used in protein synthesis and is specific for peptidoglycan synthesis (Matsuhashi *et al.*, 1967).

Once modified, the disaccharide-peptide subunit is transferred to a growing peptidoglycan chain from the lipid intermediate. Vancomycin and ristocetin are functionally related antibiotics which interrupt peptidoglycan biosynthesis by inhibiting the transfer of disaccharide-peptide subunits from the carrier lipid to the growing peptidoglycan strand. This inhibition makes it possible to study the formation and utilization of lipid intermediates separately from the rest of the reactions involved in peptidoglycan synthesis (Bordet and Perkins, 1970; Lugtenberg *et al.*, 1971). The accumulation of lipid intermediates which occurs in some systems upon exposure to vancomycin and ristocetin also facilitates their large scale production for purification and analysis. The mode of action of these antibiotics is not yet known but they apparently have a high affinity for the D alanyl-D alanine residues of the lipid linked disaccharide peptides (Bordet and Perkins, 1970).

The endogenous acceptor for the disaccharide-pentapeptide subunit was initially considered to be a growing point in the cell wall peptidoglycan (Strominger et al., 1967; Higgins and Shockman, 1971). Studies on the direction of peptidoglycan synthesis have indicated that peptidoglycan strands elongate at their reducing ends while remaining attached to the lipid intermediate (Ward and Perkins, 1973). Most peptidoglycan (70 percent) is synthesized as complete linear glycan strands at the membrane and then the entire strands are incorporated into pre-existing cell well material by crossbridging. Only 30 percent of newly synthesized peptidoglycan is incorporated directly into the pre-existing cell wall by transglycosidation (Mirelman and Sharon, 1972; Mirelman et al., 1972). This newer theory eliminates the need for a peptidoglycan "acceptor", and requires only that the "acceptor" be a glycan chain still attached to a lipid intermediate. Thus, the "acceptor" could conceivably be as small as another molecule of disaccharide-pentapeptide-P-P-carrier lipid. Peptidoglycan biosynthesis therefore proceeds by a mechanism analogous to that of O antigen biosynthesis in the outer membrane of the Gram-negative cell wall (Bray and Robbins, 1967).

Transpeptidation, the final reaction of peptidoglycan biosynthesis, frequently cannot be demonstrated in cell free systems now in use. This is especially true for the Gram-positive cocci which have complex peptidoglycan structures involving crossbridging peptides. Systems from Gram-negative organisms or Gram-positive bacilli more often contain measureable transpeptidase activity. Transpeptidation in these organisms with simpler peptidoglycan structures involves formation of a direct crossbridge between the dibasic amino acid, usually diaminopimelic acid, of one substituent peptide and the penultimate D alanine residue on a substituent peptide of an adjacent peptidoglycan strand. The formation of this peptide bond results in the release of the terminal D alanine residue. Energy necessary to drive the reaction in the forward direction is believed to come from this bond cleavage since the reaction is extracellular and removed from an endogenous supply of ATP (Tipper and Strominger, 1965).

For organisms with more complex peptidoglycan structures, where crossbridging peptides link adjacent strands together, the transpeptidation reaction is essentially the same. In these cases the bridge peptide is attached to the dibasic amino acid residue of the substituent peptide before transpeptidation occurs. Most commonly these additions are made while the disaccharide peptide subunits are still attached to the carrier lipids. Transpeptidation then involves the formation of a peptide bond between the NH_2 terminal amino acid of the bridge peptide and the penultimate D alanine residue in an adjacent substituent peptide. The terminal D alanine residue is again lost. Formation and addition of bridge peptides can be demonstrated _in vitro_ so the defect in transpeptidation for these systems must lie in the final bridge closing step.

Transpeptidation was first detected _in vitro_ using membrane fragments prepared from _E. coli_ (Araki _et al._, 1966a; 1966b; Izaki _et al._, 1968). Transpeptidation has subsequently been detected in cell free preparations from _Bacillus megaterium_ and _Streptomyces_ R39, both of which have peptidoglycan structures similar to that of _E. coli_ (Wickus and Strominger, 1972a; 1972b; Ghuysen _et al._, 1973). For Gram-positive organisms with complex peptidoglycan structures, transpeptidation has never been achieved in _in vitro_ systems when membrane fragments were used as a source of enzymes. Cell wall suspensions with associated membranous material have been successfully used to demonstrate in vitro transpeptidation for S. aureus and M. luteus (lysodeikticus) (Mirelman and Sharon, 1972; Mirelman et al., 1972). In these systems a penicillin-sensitive transpeptidase activity was demonstrated, but the synthesis of linear peptidoglycan was also somewhat sensitive to penicillin. Linear peptidoglycan synthesis was postulated to be blocked because the growing peptidoglycan strands remained attached to the membrane carrier lipids. Penicillin inhibited the transpeptidation reaction necessary to incorporate these completed strands into the cell wall. As a result, all the carrier lipid sites became saturated with completed peptidoglycan strands. In cell free systems from S. aureus and M. lysodeikticus where membrane fragments served as a source of enzymes, transpeptidation and linear peptidoglycan biosynthesis were not so closely coupled (Anderson et al., 1967). Even though transpeptidation did not occur, linear peptidoglycan was synthesized with the completed strands presumably being released free into the medium rather than incorporated into cell walls.

M. lysodeikticus and related Micrococcaceae have a complex peptidoglycan structure which contains unusual bridge peptides (Ghuysen et al., 1968; Johnson and Campbell, 1972). Unlike most peptidoglycan the bridge peptides are not simple structures made up of one or two types of amino acids nor are the bridge peptides added on at the level of the membrane bound lipid intermediates. The bridge peptides of these organisms are made up of a number of substituent peptides linked together "head to tail". Peptidoglycan biosynthesis in in vitro systems derived from these organisms results in the formation of uncrossbridged peptidoglycan molecules with each muramic acid residue bearing a substituent peptide. Crossbridge formation or assembly must therefore take place after linear peptidoglycan has been formed but before or during transpeptidation. A biosynthetic mechanism involving simultaneous amidase and transpeptidase reactions has been postulated to account for the "head to tail" assembly of these substituent peptides (Ghuysen, 1968; Schleifer and Kandler, 1967). The proposed sequence of events is as follows: two substitutent peptides (A and B) on adjacent peptidoglycan strands are joined together by transpeptidation to form a direct crosslinkage. An N-acetylmuramyl-L alanine amidase then ends this temporary crosslinkage by breaking the muramyl-L alanyl amide bond which held substitutent peptide B to the glycan backbone. Substituent peptide B remains attached to substituent peptide A and now becomes the first segment of the bridge peptide. A second transpeptidation then occurs joining a third substituent peptide (C), on another adjacent glycan strand, to the free N terminal L alanine residue of bridge peptide B. Glycan strands A and C are now crossbridged until an amidase attacks substituent peptide C and causes the whole sequence to be repeated. The cycle continues until a crossbridge with several subunit peptides has been built up. This sequence requires that complete biosynthetic systems contain an amidase as well as 2 or more transpeptidase activities. One transpeptidase activity is required to form the L lysyl-D alanyl bond which initially crosslinks substituent peptides A and B. The second transpeptidase activity is required to form the L alanyl-D alanyl bonds which join the bridge peptides together "head to tail" during bridge elongation. Final bridge closure also involves the formation of an L alanyl-D alanyl bond. These two or three transpeptidase activities may indicate that more than one transpeptidase enzyme exists, or that one enzyme with two or more specificities mediates all transpeptidation reactions. Membrane-based cell free systems from _M. lysodeikticus_ show no evidence of either amidase or transpeptidase activity (Anderson, 1967; Bordet and Perkins, 1970). Cell wall based _in vitro_ systems demonstrated transpeptidase activity by the penicillin-sensitive release of D alanine together with incorporation of labelled MurNAc-pentapeptide into cell wall material. Amidase activity was detected indirectly by finding segments of newly synthesized peptidoglycan containing only the repeating disaccharide glycan backbone with no substituent peptides. Since UDP-MurNAc cannot serve as a substrate for peptidoglycan synthesis, the unsubstituted regions were assumed to arise due to amidase activity (Mirelman _et al._, 1972).

The amidase-transpeptidase theory of crossbridge formation accounts for the "head to tail" assembly of substituent peptides into crossbridges. Control over the length of crossbridging peptides and the degree of crosslinkage could be exercised by regulating the relative activities of the transpeptidase and amidase systems. The structure of the peptidoglycan of _M. sodonensis_ has been shown to vary considerably with changing growth conditions. Peptidoglycan isolated from cells grown in complex media had relatively short crossbridges made up of 1 to 8 substituent peptides. Cells grown on a basal-salts medium had much longer crossbridging peptides ranging from 1 to 18 subunits in length (Johnson and Campbell, 1972).

Penicillin and related β lactam antibiotics function by inhibiting transpeptidation (Tipper and Strominger, 1965; Strominger _et al._, 1967). The uncrossbridged peptidoglycan which is synthesized in their presence cannot provide adequate osmotic support and cellular lysis results. Penicillin has been an invaluable aid for determining the presence or absence of a functioning transpeptidase in cell free systems. Penicillin sensitive release of D alanine when accompanied by the incorporation of in vitro synthesized peptidoglycan into pre-existing cell wall material has become a major criterion for identifying transpeptidase activity (Izaki *et al.*, 1968; Mirelman and Sharon, 1972).

A consideration of peptidoglycan biosynthesis generally centers around those reactions mentioned above which are responsible for the primary synthesis of the peptidoglycan network. A second group of enzymes referred to as autolysins degrade peptidoglycan and can cause cell lysis under certain circumstances. These enzymes were originally thought to mediate self-destructive processes. More recent evidence indicates these enzymes are frequently associated with regulation of peptidoglycan synthesis or modification of peptidoglycan structure (Rogers, 1970). Autolysins have been found in several species and likely function *in vivo* in all species even in those in which *in vitro* demonstration has been unsuccessful. Controlled lytic activity results in peptidoglycan turnover at rates as high as 30 percent per generation for some species making cell wall alteration a rapid process (Chan and Glaser, 1972; Glaser, 1973). Controlled lytic activity is also necessary to allow insertion of new material at growing points and to allow cell separation following cell division.

Many organisms contain one or more strongly lytic enzymes which will cause rapid lysis of whole cells or isolated cell walls under conditions of unbalanced growth. *Streptococcus faecalis* produces a single autolysin, an N-acetylmuramidase similar in effect to lysozyme (Shockman *et al.*, 1967a; 1967b; Pooley and Shockman, 1969). This enzyme is located in the cell wall of the organism in both active and inactive forms. Active enzyme molecules are localized at the sites of crosswall formation where net peptidoglycan biosynthesis takes place. Inactive precursors of this muramidase are located throughout the cell wall of the organism but they require modification before they become active. A net loss of peptidoglycan can only be demonstrated when cell walls are isolated from the synthetic apparatus of the cell or when whole cells are incubated under unbalanced growth conditions in a medium lacking a utilizable source of carbon and energy. Under such conditions peptidoglycan degradation takes place most rapidly in the vicinity of the growing cross walls where the active muramidase is localized. Brief treatment with trypsin converts the inactive precursor enzymes located throughout the cell wall to the active form. After such a pre-treatment cell wall degradation takes place uniformly over the entire surface of the cell wall. The muramidase of *S. faecalis* appears to regulate peptidoglycan biosynthesis and cell separation. The cell in turn controls the muramidase by storing the majority of the enzyme in an inactive form throughout the cell wall. Cell walls of *Lactobacillus acidophilus* contain an N-acetylmuramidase similar to that of *S. faecalis* but with several notable differences, (Coyette and Shockman, 1972). Unlike the muramidase of *S. faecalis*, that of *L. acidophilus* is not completely cell wall associated. As much as 25 percent of the active muramidase is located in another part of the cell. As well, no evidence of a latent form of muramidase was detected in *L. acidophilus*. These differences suggested that interconversion between latent and active forms was not a universal mechanism for controlling autolytic activity. *L. acidophilus* appears to control its potentially destructive enzyme by isolating a portion of the muramidase from the cell wall completely.

Cells of *S. aureus* are known to contain autolytic activities of at least three specificities; a glycine endopeptidase, an N-acetylglucosaminidase and an N-acetylmuramyl-L alanine amidase. These enzymes will promote lysis of isolated cell walls at a very slow rate. Approximately 24 hours was required to achieve a 50% decrease in turbidity. Analysis of the peptide products solubilized by autolysis indicated that the primary autolytic activity was an amidase. The very slow rate of autolysis of isolated cell walls indicated that during the isolation and purification of cell walls for chemical analysis, autolytic cleavage would be insignificant providing preparations were held at 4°C. Chemical analysis of cell wall peptidoglycan from *S. aureus* showed that the reducing termini of the peptidoglycan chains were almost exclusively N-acetylglucosamine residues. The known mechanism of peptidoglycan biosynthesis for all organisms should result in reducing termini made up exclusively of N-acetylmuramic acid residues. The N-acetylglucosaminidase of this organism must function extensively *in vivo* to produce these N-acetylglucosamine reducing termini. The amidase activity which is primarily responsible for autolysis of isolated cell walls had only minimal effects *in vivo* on peptidoglycan structure since only 7 percent of the L alanine residues have free NH_2 groups (Tipper, 1969).

Cells of *E. coli* like those of *S. aureus* also contain a wide range of peptidoglycan-hydrolysing enzymes including a muramidase, a glucosaminidase, a muramyl-L alanine amidase, several endopeptidases and 2 or more carboxypeptidases (Hartmann *et al.*, 1974; Hakenbeck *et al.*, 1974). These enzymes are located within the membrane rather than the cell wall and are under very tight control. The activity of some of the hydrolases is regulated both spatially and temporally so that localized hydrolase action is triggered at a given stage of cell division (Schwarz *et al.*, 1969). Within the intact cells, no evidence of any hydrolytic activity can be detected suggesting that a barrier may exist which separates the enzymes from their substrate *in vivo*. In *E. coli* this barrier can be disrupted by mechanically opening the cells or by exposing whole cells to NaCl, EDTA, or 5 percent TCA. This concept of a barrier seems applicable to the growing number of organisms known to contain lytic enzymes which are not expressed *in vitro*, and may represent a more widespread mechanism of regulating peptidoglycan hydrolase activity.

Non-peptidoglycan components of bacterial cell walls make up 10 to 50 percent of the Gram-positive cell wall and 85 to 95 percent of the Gram-negative cell wall. Many of these compounds are polysaccharides or sugar-containing polymers. Biosynthesis of these compounds has been found to closely parallel peptidoglycan biosynthesis involving activation of the component sugar residues as nucleotide precursors, and transport through the membrane for assembly by means of carrier lipids.

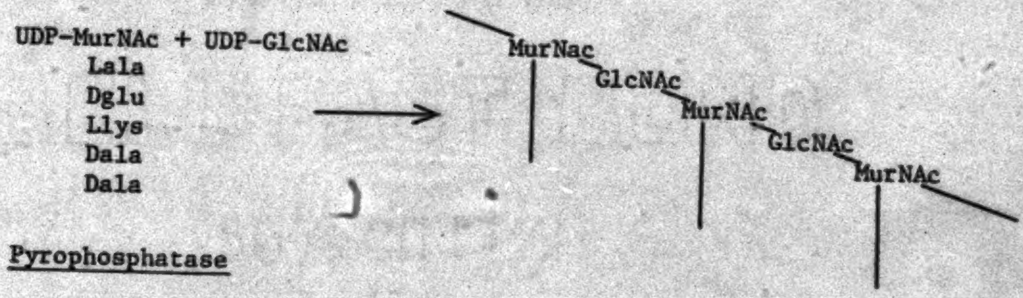
The synthesis of polymeric cell envelope constituents as diverse as peptidoglycan, wall techoic acids, lipopolysaccharide (O antigen) of the outer membrane of Gram-negatives, and several extracellular polysaccharides all involve use of carrier lipids (Anderson *et al.*, 1973; Baddiley, 1972; Lenarz and Scher, 1972; Warren and Jeanloz, 1972; Strominger *et al.*. 1972).

The carrier lipid involved in peptidoglycan biosynthesis has been isolated and identified as a C_{55} isoprenoid alcohol, called undecaprenol (Higashi et al., 1967). Preliminary investigations of the carrier lipid of O antigen synthesis indicates it to be a polyisoprenoid compound with a structure similar to or the same as undecaprenol (Nikaido, 1968). Carrier lipids for techoic acid biosynthesis are present in such small amounts that they have not been isolated or characterized. These carrier lipids were however indirectly shown to be identical to undecaprenol by the development of particulate preparations from S. lactis capable of synthesizing peptidoglycan and glycerol techoic acids at the same time. The two synthetic systems were found to compete with each other for the limited supply of carrier lipid molecules. Nucleotide precursors for peptidoglycan would inhibit the techoic acid synthesizing system and conversely nucleotide precursors for techoic acid would inhibit peptidoglycan biosynthesis. A similar situation has also been demonstrated in Bacillus licheniformis (Baddiley, 1972; Anderson et al., 1973).

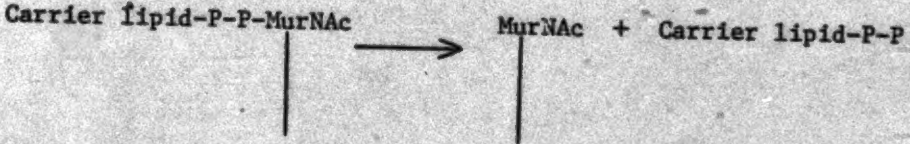
The inter-relationships between biosynthetic mechanisms for the synthesis of these extracellular cell wall components suggests another way in which peptidoglycan biosynthesis could be controlled. The availability of carrier lipid molecules in the active monophosphorylated form determines the extent of peptidoglycan synthesis and possibly the amount of peptidoglycan produced could be regulated by altering the rates of synthesis of competing compounds. In a more direct approach the synthesis of all carrier lipid dependent extracellular products could be regulated by controlling the activity of C_{55} isoprenylpyrophosphatase and related enzymes. These enzymes regenerate the used carrier lipid molecules from the pyrophosphate form to the active monophosphate form. Rudiments of such a control mechanism have been detected in *S. aureus* and their *in vivo* effectiveness is being investigated (Strominger *et al.*, 1972).

This investigation was undertaken to examine peptidoglycan biosynthesis as it occurs in cell free preparations of *M. sodonensis*. Peptidoglycan biosynthesis is a multi-step process involving an undetermined number of enzymatic conversions. Some of the activities involved are indicated schematically below.

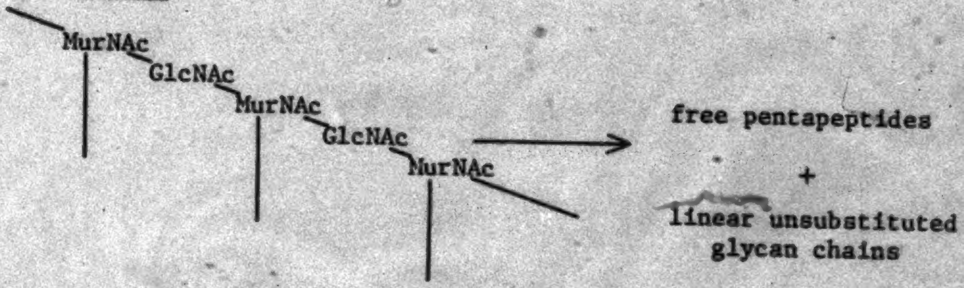
1. **Transglycosidase**


UDP-MurNAc + UDP-GlcNAc
Lala
Dglu
Llys
Dala
Dala
MurNac
GlcNAc
MurNAc
GlcNAc
MurNAc

2. **Pyrophosphatase**


Carrier lipid-P-P-MurNAc
MurNAc + Carrier lipid-P-P

3. **Amidase**


MurNAc
GlcNAc
MurNAc
GlcNAc
MurNAc
free pentapeptides
+
linear unsubstituted glycan chains

4. **Transpeptidase**

linear glycan chains → crosslinked glycan network

For the purposes of this investigation, transglycosidase activity, 1., was defined as the overall process which results in conversion of UDP-GlcNAc and UDP-MurNAc-pentapeptide into uncrosslinked peptidoglycan. Pyrophosphatase activity, 2., is defined as the enzyme which mediates the overall conversion of UDP-MurNAc-pentapeptide to MurNAc-pentapeptide and UMP. This, however appears to be an indirect process in which carrier lipid-P-P-MurNAc-pentapeptide is the actual substrate for pyrophosphatase activity. Carrier lipid-P-P-MurNAc-pentapeptide is an intermediate in transglycosidation and is formed by that system with the concomitant release of UMP. This lipid intermediate can by incorporated into peptidoglycan if conditions permit, but if peptidoglycan biosynthesis is prevented then it can be acted upon by pyrophosphatase to yield MurNAc-pentapeptide and carrier lipid-P-P. The carrier lipid is then regenerated to the monophosphorylated form as it is in transglycosidation. Amidase activity, 3., cleaves some substituent peptides off of the linear peptidoglycan so that they may participate in crossbridge formation. In the absence of transpeptidase activity however, amidase activity causes the release of free pentapeptides. Transpeptidase activity, 4., joins the cleaved substituent peptides together "head to tail" to form the crossbridging peptide and finally completes bridge closure by joining adjacent peptidoglycan strands together.

Activities 1.,2., and 3., have been detected in an *in vitro* system prepared from *M. sodonensis* and will be described in greater detail. Attempts were also made to relate the enzyme activities detected with those known to exist in cell free systems from other organisms.

MATERIALS AND METHODS

I. Reagents, Substrates etc.

UDP-MurNAc-^{14}C-pentapeptide was prepared and purified as described in part VI of this section. Highly purified cell walls, isolated from stationary-phase cells of *M. sodonensis*, were provided by Dr. K. G. Johnson. All chemicals used in this investigation were of reagent grade and were purchased from commercial sources indicated. Enzymes were prepared as described or purchased commercially from the sources indicated.

II. Culture and Growth Conditions

A. *Growth Medium*. Trypticase Soy Broth (TCS), (Baltimore Biological Laboratories) pH 7.3 was used in all cases as the liquid growth medium. Bacto-agar (Difco) was added to yield 1.5% (w/v) when a solid medium was required.

B. *Culture*. *Micrococcus sodonensis* ATCC 11880 was the organism used throughout this study. Stock cultures were stored in lyophilized form and also maintained on TCS agar plates stored at 4°C with monthly transfers. All cultures were grown aerobically at 30°C. Liquid cultures containing 400ml of media in 2 l flasks were aerated on a New Brunswick Gyrotory Shaker operating at 330 rpm.

Cells for membrane and cell wall preparation were grown by inoculating 1.2 l amounts of TCS broth to 0.1% (v/v) with a stationary-phase culture of *M. sodonensis*. Cultures were incubated 11 hours for production of exponential-phase cells and 18 hours for production of stationary-phase cells. Resulting optical densities at 600 nm were approximately 15 and 45 respectively, as measured on appropriately diluted samples.

III. Analytical Methods

A. Protein. Protein content of membrane suspensions and soluble amidase preparations was measured by the method of Lowry (Lowry *et al.*, 1951), after removal of β mercaptoethanol by dialysis or dilution. Protein content of cell wall suspensions was estimated by modification of the Lowry method as described by Mirelman (Mirelman *et al.*, 1971).

B. Carbohydrates.

1. Dextran was measured by the anthrone technique using Dextran T-10 (Pharmacia Corporation) as a standard (Scott and Melvin, 1953).

2. Total hexosamines were determined by the Morgen-Elson reaction as modified by Ghuysen (Ghuysen *et al.*, 1966). Samples containing 10 to 50 nmoles of hexosamine were hydrolysed in sealed tubes in 3N HCl at 100°C for 3 hours. The free hexosamines were acetylated by exposure to aqueous acetic anhydride under alkaline conditions. Once acetylated, the samples were boiled for 7 min in borate and then reacted with p dimethylamino benzaldehyde under strongly acid conditions. Color development proceeded for 20 min at 37°C and absorbance at 585 nm was measured. Standard N-acetylglucosamine samples were subjected to the complete hydrolysis and color development procedures.

3. Soluble N-acetylhexosamines were determined by a second modification of the Morgen-Elson reaction as described by Strominger (Strominger, 1957). N-acetylglucosamine was again employed as a standard. Standards and test samples were exposed to a 5 min hydrolysis in 0.125 N HCl at 100°C. N-acetyl groups survive this brief hydrolysis period so chemical re-

acetylation was not required.

4. Reducing sugars were determined by the modified Park-Johnson ferricyanide procedure (Ghuysen _et al._, 1966).

5. Muramic acid residues with free reducing groups were quantitated by reduction with tritiated $NaBH_4$ (New England Nuclear) and isolation of the resulting amino sugar alcohols in a modification of the original procedure as described by Hughes (Hughes, 1970). One μmole amounts of glucosamine and muramic acid were used as standards. Standard and test samples were treated with an excess of 1% (w/v) $NaBH_4$ (0.4 ml; approximately 2.43 μCi/μmole) in 50 mM sodium borate buffer, pH 8.9 at 35°C for 16 hours. Excess $NaBH_4$ was destroyed by the addition of 0.2 ml of 12 N HCl. The acidified samples were sealed and hydrolysed for 3 hours at 100°C, then dried _in vacuo_. The residues were then dried several times from water and methanol to remove the remaining methyl borate. Samples were finally resuspended in 0.5 ml of water for chromatography on Dowex 50 to separate the radioactive amino sugar alcohols.

C. _Total Amino Acids_. Total amino acids were determined as their dinitrophenyl derivatives (Ghuysen, 1966). Samples containing approximately 30 nmoles of each amino acid were hydrolysed in sealed tubes in 6 N HCl at 100°C for 18 hours. DNP-amino acid derivatives were formed by the reaction of the amino acid-containing hydrolysates with 1-fluoro-2,4-dinitrobenzene (Fisher) under alkaline conditions at 60°C for 1 hour. DNP derivatives were extracted into diethyl ether, dried, and spotted onto thin layer plates of silica gel. The plates were developed in solvents C and D as described elsewhere and the separated derivatives were collected, eluted with 2.0 ml of ethanol: water: ammonia (370:370:2; v/v), and centrifuged to remove suspended silica gel. Absorbance at 360 nm of the resulting supernatants was measured in a Gilford 240 Spectrophotometer. Standard amino acid mixtures were subjected to the complete acid hydrolysis and derivatization procedure in parallel with the test samples.

D. N-terminal Amino Acids. Estimation of the total amount of free N-terminal alanine was carried out as described by Ghuysen (Ghuysen, 1966). The method was essentially the same as for estimation of total amino acid composition except that the derivitization step preceeded acid hydrolysis.

IV. Chromatographic Techniques

A. Developing Solvents. (v/v)

1. Solvent A- isobutyric acid: 0.5 N ammonia (5:3) (Bordet and Perkins, 1970).
2. Solvent B- ethanol: 1 M ammonium acetate (5:2) (Bordet and Perkins, 1970).
3. Solvent C- n butanol saturated with 1% ammonia (Ghuysen, 1966).
4. Solvent D- chloroform: methanol: acetic acid (85:14:1) (Ghuysen, 1966).

B. Paper Chromatography. Descending chromatography with 4 cm x 55 cm strips of Whatman 3 MM paper was used in all cases.

1. Separation of Compounds

   a. Uridine nucleotides, peptidoglycan and lipid intermediates were separated by development of chromatograms in solvent A.

   b. Uridine nucleotides could also be separated by development of chromatograms in solvent B.

2. Location of Compounds

a. Nucleotides and derivatives were located by examining the paper strips under ultraviolet light with a wavelength of 260 nm.

b. Radioactivity was located on the paper strips by means of a Nuclear Chicago Actigraph III Strip Scanner. For quantitation of radioactivity, the paper strips were cut into 1 cm sections, immersed in 5 ml of a toluene based scintillation fluid and counted in a Nuclear Chicago Mark I Scintillation Counter.

C. Thin Layer Chromatography. Thin layer plates of MN-Silica Gel G-HR (Macherey, Nagel and Co.) were prepared on glass plates 20 cm x 20 cm, and activated at 100°C for 1 hour before use. DNP-amino acid derivatives were dissolved in a minimal amount of methanol and then spotted onto the silica gel plates. Nα-mono DNP-amino acids and Nαε-di DNP-lysine were partially resolved by developing the thin layer plates halfway in solvent C. The plates were then thoroughly dried, and the DNP derivatives were further separated by developing the dried plates completely in solvent D (Ghuysen, 1966).

D. Ion Exchange Chromatography. Dowex ion exchange resins were obtained from Bio-Rad Laboratories. Dowex 50W- X4 (200-400 mesh) in the H^+ form was employed for the separation of muramitol from glucosaminol and methyl borate. One cm x 20cm columns of resin, equilibrated with 0.1 M pyridine acetate buffer, pH 2.8, were set up in a well ventilated area. The buffer was 0.1 M with respect to pyridine and contained sufficient acetic acid to lower the pH to 2.8 (Brendel et al., 1967). Samples dissolved in 0.5 ml of water were applied to the columns and eluted first with 70 ml of 0.1 M pyridine acetate buffer, pH 2.8, to remove the methyl borate and muramitol, and then with 70 ml of 0.133 M pyridine acetate buffer, pH 3.85, to remove glucosaminol.

E. Gel Filtration. Sephadex gels and defined dextran fractions were obtained from Pharmacia Corp. Gel filtration properties of compounds are expressed in terms of the distribution coefficient, Kay.

$$Kav = \frac{Ve - Vo}{Vt - Vo}$$

Ve = elution volume of the compound; Vo = void volume of the system; and Vt = total volume of the system.

Void volumes were determined using a 1 mg/ml solution of Dextran Blue 2000 and monitoring effluent fractions for absorbance at 620 nm. Total volume of Sephadex G-200 columns was established using a 1 mg/ml solution of N αε-di DNP lysine and monitoring effluent fractions for absorbance at 360 nm. Total volume for Sephadex G-15 and G-25 columns was established using 1 M NaCl. Effluent fractions were monitored for Cl^- with saturated $AgNO_3$.

1. Desalting. Materials to be desalted were eluted from calibrated Sephadex G-15 or G-25 columns with distilled water. Conductivity of effluent fractions was determined by means of a Type CDM 2 Bach Simpson conductivity meter.

2. Purification of Small Molecular Weight Compounds. UDP-MurNAc-^{14}C-pentapeptide, MurNAc-^{14}C-pentapeptide, and free ^{14}C-pentapeptide were purified in part by gel filtration on Sephadex G-25 columns (2.5 x 95 cm). The columns were equilibrated and eluted with water at an operating pressure of 1.5 m. Five ml fractions were collected at a rate of 70 ml per hour.

3. Calibration of a Sephadex G-200 Column. A Sephadex G-200 column (2.5 cm x 45 cm) was set up and equilibrated with 0.3% NaCl at 4°C. The column was run in the reverse direction by means of an upward flow adaptor at an operating pressure of 10 cm and a flow rate of 20 ml per hour. Calibration was achieved by chromatographing a series of dextran standards. Two mg amounts of each of Dextran T-10, T-20, T-40, T-70, T-110 and T-150 were applied separately in 1 ml volumes and effluent fractions were monitored for carbohydrate. The distribution coefficient of each dextran standard was correlated with its known average molecular weight.

V. Electron Microscopy

Samples for microscopy were supported on carbon-shadowed, Formvar-coated, copper grids, negative contrast stained with 3% (w/v) phosphotungstic acid and examined in a Phillips Model 200 Electron Microscope.

VI. Preparation of UDP-MurNAc-^{14}C-pentapeptide

Radioactively labelled UDP-MurNAc-^{14}C-pentapeptide was prepared by a modification of the method of Garrett (Garrett, 1969). Three hundred ml amounts of TCS broth containing 1% (w/v) sodium lactate were inoculated to 10% (v/v) with a stationary-phase culture of *M. sodonensis* also grown in the same medium. The cultures were incubated 3.5 hours at 30°C until cells reached the mid-exponential phase of growth (OD_{600} = 13). The cells were harvested by centrifugation and resuspended into 250 ml amounts of 50 mM sodium phosphate buffer, pH 7.0, containing 1 mM EDTA, 0.005% (w/v) chloramphenicol, and 1% (w/v) sodium lactate. After 5 min incubation, 10 µCi each of ^{14}C-uniformly labelled L alanine (157 mCi/mmole, New England Nuclear) and ^{14}C-uniformly labelled D alanine (36 mCi/mmole, Amersham Searle) were added and mixed. After another 5 min incubation, unlabelled glutamic acid, lysine and alanine were added to a final concentration of 0.002% (w/v). The flask was incubated aerobically at 30°C for 1 hour. The cells were again harvested by centrifugation and the cell pellet was resuspended in a minimal amount of distilled water. After cooling to 4°C, 25 ml of ice cold 25% TCA was added. The acidified cells were held for 30 min at 4°C and then the precipitated material was centrifuged out. The pellet was again resuspended in 25 ml of ice cold 25% TCA and the extraction procedure was repeated. The two supernatants were pooled, and extracted with 3 x 50 ml volumes of diethyl ether to remove TCA, and neutralized. The neutralized extract was lyophilized and UDP-MurNAc-^{14}C-pentapeptide was purified by a combination of gel filtration on Sephadex G-25 and paper chromatography.

VII. Enzyme Assays

A. Transglycosidase. The reaction mixture contained 0.875 M Tris HCl, pH 8.6, 20 µl; 0.1 M $MgCl_2$, 10 µl; 2 mM UDP-GlcNAc, 10 µl; and 0.8 mM UDP-MurNAc-^{14}C-pentapeptide, 15 µl (Bordet and Perkins, 1970). Either 70 µl of membrane suspension, vide infra (7 mg protein/ml = 0.5 mg protein), or 70 µl of cell wall suspension, vide infra (1mg protein/ml = 0.07 mg protein) was added as an enzyme source. The final reaction volume was 140 µl. Reaction mixtures were incubated 3 hours at 30°C and then boiled for 1 min to stop the reaction. Reaction mixtures were streaked onto paper strips and developed for 16 hours in solvent A, dried, washed with acetone (to remove the isobutyric acid) and scanned for radioactivity. Radioactive material remaining at the origin was considered the product of transglycosidase activity.

B. Transpeptidase. The reaction mixture contained 0.9 M Tris HCl, 20 µl; 0.9 M NH_4Cl, 20 µl; 0.36 M $MgCl_2$, 10 µl; 0.27 M ATP, 10 µl; 0.018 M β mercaptoethanol, 10 µl; 0.05 M glycine, 10 µl; 2 mM UDP-GlcNAc, 10 µl; and 0.8 mM UDP-MurNAc-^{14}C-pentapeptide, 15 µl (Mirelman and Sharon, 1972). Again, either 70 µl of membrane suspension (0.5 mg of protein) or 70 µl of cell wall suspension (0.07 mg protein) served as a source of enzymes. Assays were incubated 3 hours at 30°C and boiled 1 min to stop the reaction. Reaction products were separated and quantitated as in the transglycosidase assay. The presence of free ^{14}C-alanine (Rf of 0.65) was taken as an indication of transpeptidase activity.

C. Pyrophosphatase. Maximum pyrophosphatase activity could be obtained using the standard transglycosidase assay by omitting the UDP-GlcNAc and modifying the concentration of reagents as follows: 0.875 M Tris HCl, pH 8.6, 20 µl; 0.6 M $MgCl_2$, 10 µl; and 0.8 mM UDP-MurNAc-^{14}C-pentapeptide, 15 µl. This standard assay was performed using 70 µl of membrane suspension (0.5 mg of protein) in a final reaction volume of 140 µl. Assays were incubated 3 hours at 25°C and boiled 1 min to stop the reaction. Radioactivity chromatographing with an Rf of 0.6 was taken as a measure of pyrophosphatase activity.

D. Amidase. Two types of assays were used to measure N-acetylmuramyl-L alanine amidase activity.

1. In a type 1 amidase assay the amidase containing sample was added to a completed transglycosidase assay which had been inactivated by boiling for 1 min. Stationary-phase membranes served as the enzyme source for the transglycosidase assay. The amidase-containing transglycosidase system was again incubated 3 hours at 30°C and boiled for 1 min

to inactivate the amidase. The stopped reaction mixture was streaked onto a paper strip and chromatographed in solvent A. Disappearance of radioactivity from the origin when compared to a control transglycosidase assay, or appearance of radioactivity at an Rf of 0.65 was taken as a measure of amidase activity.

2. In a type 2 amidase assay, partially purified peptidoglycan replaced the crude transglycosidase system as a source of *in vitro* synthesized peptidoglycan. Tris HCl was added to give a final concentration of 0.125 M at a pH of 8.6 and $MgCl_2$ was added to give a concentration of 7 mM. The reaction mixture was incubated 3 hours at 30°C and stopped by heating to 100°C for 1 min. Reaction products were separated and quantitated by the same method used in the type 1 assay.

E. *Lysozyme*. *In vitro* synthesized peptidoglycan was digested by the addition of 100 μg of egg white lysozyme (Sigma) to a stopped transglycosidase reaction mixture. Sodium azide was added to 0.02% (w/v) in a final reaction volume of 250 μl and at a final pH of 8.6. After 20 hours incubation at 37°C the digestion products were separated by paper chromatography in solvent A.

F. *Succinic dehydrogenase*. Activity was followed by measuring the decrease in absorbance at 400 nm of an enzyme system containing 0.05 M $MgSO_4$, 0.1 ml; 0.1 M KCN, 0.1 ml; 0.01 M $K_3Fe(CN)_6$, 0.03 ml; 0.01 M phenazine methosulfate, 0.02 ml; 0.1 M disodium succinate, 0.1 ml; 0.2 M sodium phosphate buffer, pH 7.0, 0.4 ml; and from 0.1 to 0.25 ml of enzyme. A change in absorbance of 1.0 at 400 nm was equivalent to 0.77 μmoles of succinate oxidized.

G. *NADH dehydrogenase*. Activity was followed by measuring decrease in absorbance in a system containing 0.1 M sodium phosphate buffer, pH 7.2, 0.5 ml; 0.001 M NADH, 0.2 ml; 0.001 M triphenyl tetrazolium chloride, 0.05 ml; and 0.1 to 0.25 ml of enzyme solution. A decrease in absorbance at 340 nm of 1.0 was equivalent to 0.235 µmoles of NADH being oxidized.

VIII. Production of Cell Membrane Suspensions

Exponential-phase or stationary-phase cells as required were washed once in 50 mM Tris HCl, pH 7.5, containing 1 mM β mercaptoethanol (TME buffer), and then broken by one of the following three methods.

A. Grinding With Plastic Beads. Washed cell pellets were resuspended in a minimal amount of TME buffer to yield a thick cell slurry. Equal weights of cell slurry and plastic beads (Bio Beads S-X8, 200-400 mesh, BioRad Laboratories) were combined in a Sorvall Omni-mixer. The cells and beads were then mixed at maximum speed for 2 to 10 min. The broken cell slurry was diluted with TME buffer. Plastic beads, whole cells and cell walls were sedimented by centrifugation for 3 X 5 min at 15,000 xg. Membrane fragments were sedimented from the supernatant by centrifugation for 45 min at 48,000 xg. The membranes were washed twice in TME buffer and resuspended to 7 mg protein per ml for use. All manipulations were carried out at 4°C unless otherwise indicated.

B. Grinding With Alumina. A pellet of washed cells was vigorously ground with 3 times its wet weight of levigated alumina in a pre-cooled mortar and pestle for 10 min at 4°C. The cells-alumina mixture was diluted out with TME buffer and then treated exactly as the broken cell slurry from grinding cells with plastic beads.

C. Lysozyme-French Press. Washed cells were resuspended in TME buffer and exposed to lysozyme at 100 µg/ml for 1 hour at 30°C. The viscous pellet was forced through a French pressure cell at 15,000 pounds per square inch pressure. The resulting broken cell slurry was diluted out with TME buffer and treated exactly as the broken cell slurry from grinding cells with plastic beads.

IX. Production of Cell Wall Suspensions

Cell walls were prepared from 1.2 l batches of cells grown in the same manner as for cell membrane production. Cell breakage was achieved by grinding a cell slurry with plastic beads in an Omni-mixer for 2 min at maximum speed. Cell walls were separated from plastic beads, whole cells and cell membranes by differential centrifugation. A series of 10 min centrifugations at 1000 xg was continued until all plastic beads and whole cells had been sedimented. Usually five or six centrifugations were required. Cell walls were then sedimented by centrifugation at 8000 xg washed twice in TME buffer and then resuspended to 1 mg protein per ml for use.

X. Solubilization of Enzymes

Three methods were used in attempts to release bound enzymes from cell walls or membranes. In each case the membrane suspensions contained 7 mg of protein per ml and the cell wall suspensions contained 1 mg of protein per ml.

A. Triton X-100. Membrane or cell wall suspensions were made 1% (v/v) with respect to Triton X-100 (Rohm and Haas). After brief mixing the suspensions were centrifuged at 48,000 xg for 45 min to sediment insoluble material (Umbreit and Strominger, 1973).

B. Butanol Extraction. Membrane or cell wall suspensions were mixed vigorously with equal volumes of water saturated n butanol and held at 4°C for 15 min with periodic mixing. The emulsion was then centrifuged at 48,000 xg for 45 min to separate the phases and sediment insoluble material. The upper butanol layer and interface material were discarded. the aqueous layer and pellet were saved (Barnett, 1973).

C. LiCl Extraction. Membranes or cell walls were sedimented from suspension by centrifugation at 48,000 xg for 45 min. The pellets were then resuspended to their original volumes in 6 M LiCl containing 1 M Tris HCl, pH 8.0, and held at 4°C for 60 min. The membranes and cell walls were again sedimented by centrifugation at 48,000 xg for 45 min (Pooley *et al.*, 1970).

For each of these three methods the insoluble pellets of membrane and cell wall material were resuspended to their original volume in TME buffer. Both the resuspended pellets and aqueous extracts were dialysed for 16 hours at 4°C against TME buffer before enzyme activity was measured. If protein content was to be measured, buffer lacking β mercaptoethanol was used for dialysis. Seventy μl amounts of each extract or resuspended pellet was assayed for enzyme activity using a standard assay.

EXPERIMENTAL

I. Preparation of UDP-MurNAc-^{14}C-pentapeptide

UDP-MurNAc-pentapeptide is a necessary substrate in any *in vitro* peptidoglycan synthesizing system. It was originally observed to accumulate in cells of *S. aureus* which had been exposed to penicillin. Addition of penicillin to cultures of *M. sodonensis* at 5 μg/ml (approximately 10 x MIC) caused no accumulation of hexosamine containing nucleotides. Cation deprivation had also been observed to induce intracellular accumulation of nucleotide precursors in *S. aureus* and in a wide range of other organisms as well (Garrett, 1969). By modifying the growth medium, this technique was used to prepare UDP-MurNAc-^{14}C-pentapeptide from *M. sodonensis*. The incubation mixture contained EDTA to produce the cation deficiency, ^{14}C-uniformly labelled D and L alanine and chloramphenicol to prevent incorporation of the labelled L alanine into protein. TCA extraction was used to release the nucleotide precursor from the cells and the neutralized extract was applied to a Sephadex G-25 column. Fractions were assayed for radioactivity, absorbance at 260 nm, and soluble hexosamines (Figure 1).

Two main peaks of hexosamine containing material were detected but only one was radioactive, (Peak I). The second hexosamine containing compound, (Peak II) was UV-absorbing but analysis showed that it contained no amino acids. It was probably UDP-GlcNAc or unsubstituted UDP-MurNAc. The radioactive peak with a Kav of 0.98 (Peak III) was demonstrated to be free alanine. Radioactive, hexosamine containing fractions were pooled and lyophilized. The dried material was streaked on paper strips at a rate of 0.5 μmoles of hexosamine per cm and

FIGURE 1

GEL FILTRATION (SEPHADEX G-25) OF PRODUCTS ACCUMULATED BY CATION DEPRIVED CELLS OF *M. SODONENSIS*

Five ml of neutralized TCA extract from cation deprived cells was applied to a Sephadex G- 25 column (2.5 x 95 cm) and eluted with water. Five ml fractions were collected and then UV absorption, total radioactivity, and total soluble hexosamine content of each fraction was measured.

— — — radioactivity

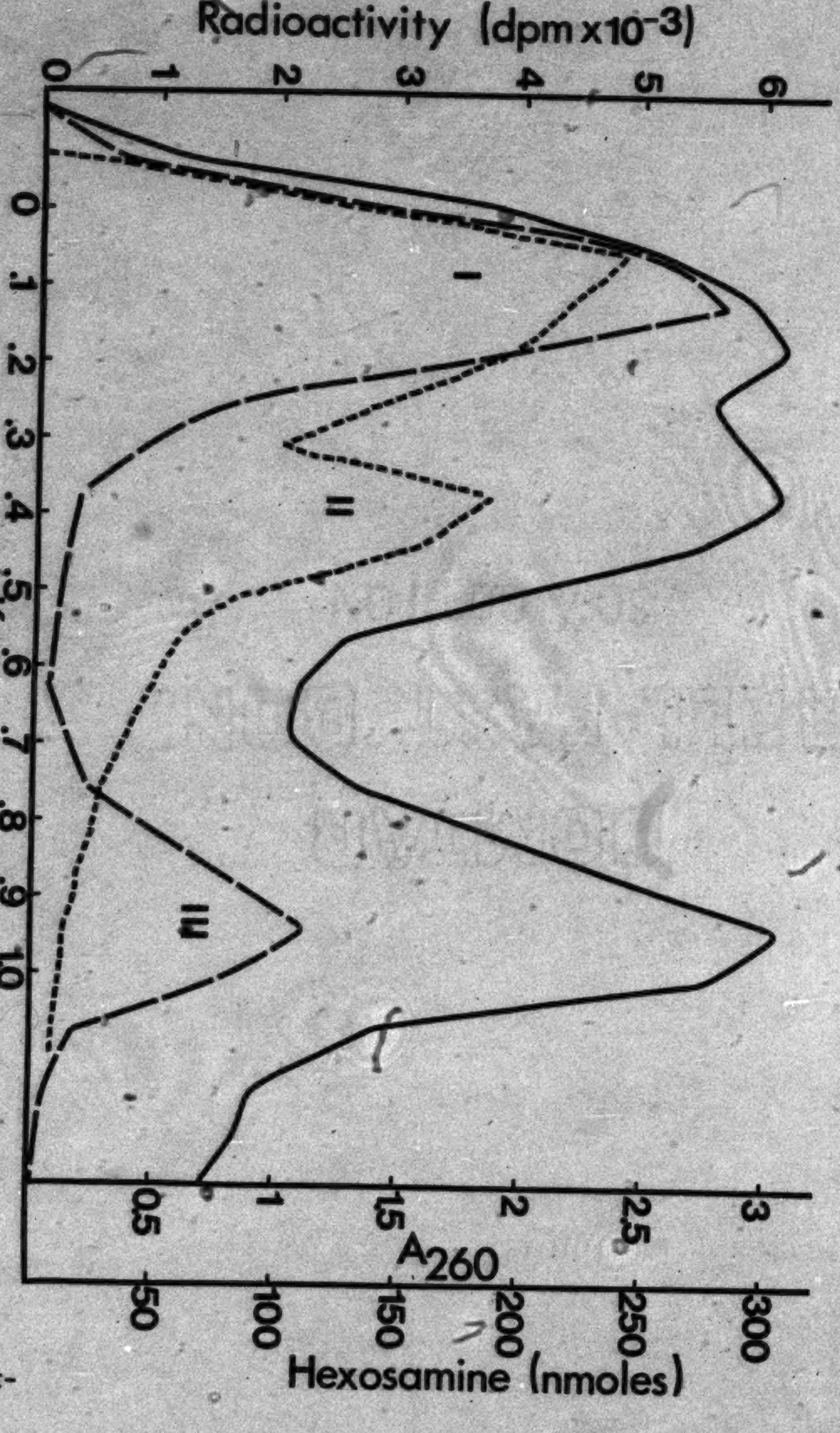
·················· soluble hexosamine

———— absorbance at 260 nm

I = UDP-MurNAc-^{14}C-pentapeptide

II = UDP-MurNAc or UDP-GlcNAc

III = free ^{14}C alanine


Radioactivity (dpm x10^{-3})
0
1
2
3
4
5
6
Kav
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
I
II
III
A$_{260}$
0.5
1
1.5
2
2.5
3
Hexosamine (nmoles)
50
100
150
200
250
300

then chromatographed for 18 hours in solvent A. Radioactivity was located using a strip scanner. The scanning speed was 60 cm/sec at a sensitivity setting of 1000 cpm and the position of the paper strip on the resulting printout was determined by using a radioactive marker dye (Figure 2). The only major radioactive band seen (Rf = 0.325) corresponded with an intensely UV-absorbing spot. This positive band from each strip was eluted extensively with water until all radioactivity had been removed. The eluant was lyophilized, applied to paper strips and chromatographed in solvent B for 11 hours. Again only one radioactive, UV-absorbing band was present, in this case at an Rf of 0.125. The material was eluted from the paper strips, concentrated and desalted. The final purified product was concentrated to a volume of 10 ml and assayed for soluble N-acetyl hexosamines and total amino acids (Table I). The final yield of product was 5.135 x 10^6 dpm in 7.4 μmoles of UDP-MurNAc-^{14}C-pentapeptide (based on soluble hexosamine content) for a specific activity of 694 dpm per nmole. The material contained hexosamine, glutamic acid, lysine, and alanine in a molar ratio approaching 1:1:1:3. No glycine was detected in the material.

II. Transglycosidase Activity of Stationary-phase Membrane Suspensions

A. Preparation of Membrane Suspensions.

Membrane suspensions capable of carrying out transglycosidation were prepared from stationary-phase cells of *M. sodonensis*.

1. Effect of Methods of Cell Breakage on Transglycosidase Activity. Whole cells of *M. sodonensis* are extremely resistant to breakage by the usual mechanical means. As a result, fairly harsh methods of cell breakage

FIGURE 2

PAPER CHROMATOGRAPHY OF PARTIALLY PURIFIED UDP-MurNAc^{14}C-pentapeptide

Two µmoles of partially purified UDP-MurNAc-^{14}C-pentapeptide (based on soluble N-acetylhexosamine content) were applied to paper strips and developed 18 hours in solvent A. Radioactive material was located using a strip scanner.

I = UDP-MurNAc-^{14}C-pentapeptide

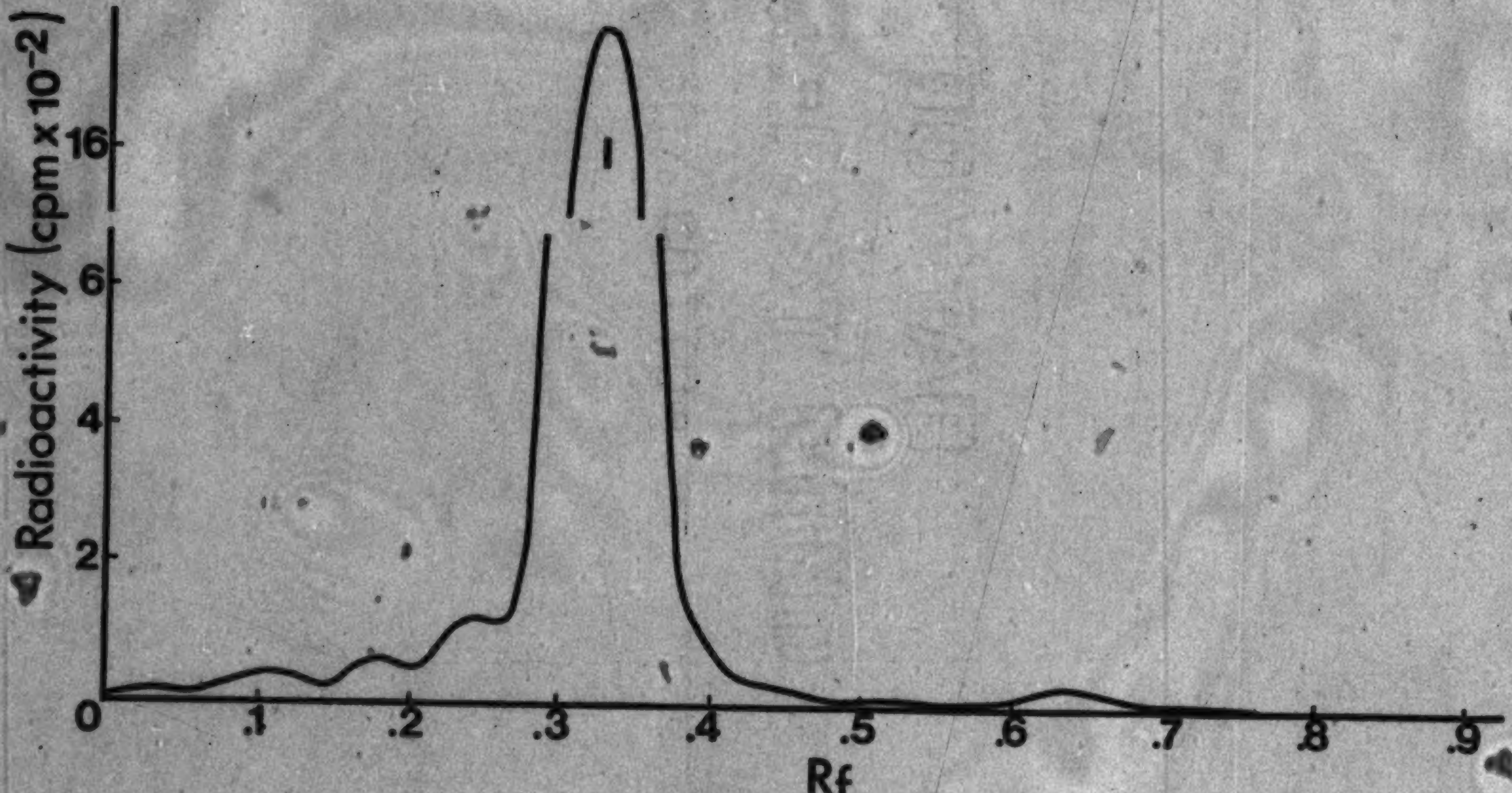

Radioactivity (cpm x 10^{-2})
0
2
4
6
16
I
.1
.2
.3
.4
.5
.6
.7
.8
.9
Rf

TABLE I

COMPOSITION OF UDP-MurNAc-^{14}C-pentapeptide (PEPTIDOGLYCAN PRECURSOR) ACCUMULATED BY CATION DEPRIVED CELLS OF M. SODONENSIS

Component	Amount[a]	Molar Ratio
Soluble N-acetyl-hexosamine	740	1.00
Glutamic acid	732	0.99
Glycine	b	b
Alanine	2340	3.20
Lysine	770	1.04

a nmoles per ml.

b Below levels of detection

had to be employed. Unfortunately, these methods frequently partially destroyed transglycosidase activity.

a. Grinding With Plastic Beads. A thick slurry of washed stationary-phase cells of M. sodonensis was ground with an equal weight of plastic beads in an Omni-mixer at maximum speed for 2 min. This method represented a successful compromise between efficient cell breakage and maintenance of enzyme activity. Cell breakage was restricted to about 30 percent which resulted in a moderate yield of membrane fragments with a high level of transglycosidase activity (Figure 3). The peptidoglycan product formed during a normal transglycosidase assay using these membrane suspensions remained at the origin of chromatograms developed in solvent A. The residual labelled substrate (Peak II) moved to an Rf of 0.3. Small amounts of lipid precursors were present near the solvent front. Peak III material, which consisted of a mixture of degraded substrate (MurNAc-pentapeptide), and degraded peptidoglycan was present at an Rf of 0.6 to 0.65. Under optimum conditions these membrane suspensions prepared by grinding cells with plastic beads for 2 min could promote the incorporation of 4.7 nmoles of MurNAc-^{14}C-pentapeptide into peptidoglycan per hour per mg of membrane protein. Increasing the grinding time to 10 min gave 100 percent breakage but completely destroyed enzyme activity. Since grinding with plastic beads for short periods as a method of cell breakage yielded membrane suspensions with consistently high levels of enzyme activity, this method was routinely used for all subsequent studies on enzyme activities associated with the cell membrane. Membrane suspensions produced by this method were examined with an electron microscope. The membranes were negative-contrast stained with phosphotungstic acid (Plate I). The membrane fragments ranged in

FIGURE 3

TRANSGLYCOSIDASE ACTIVITY OF STATIONARY-PHASE MEMBRANE SUSPENSIONS OF *M. SODONENSIS* PREPARED BY GRINDING CELLS WITH PLASTIC BEADS

One hundred forty µl of transglycosidase reaction mixture was applied to a paper strip and chromatographed 16 hours in solvent A. Distribution of radioactive products was determined by cutting the strip into 1 cm sections and counting each section in a scintillation counter.

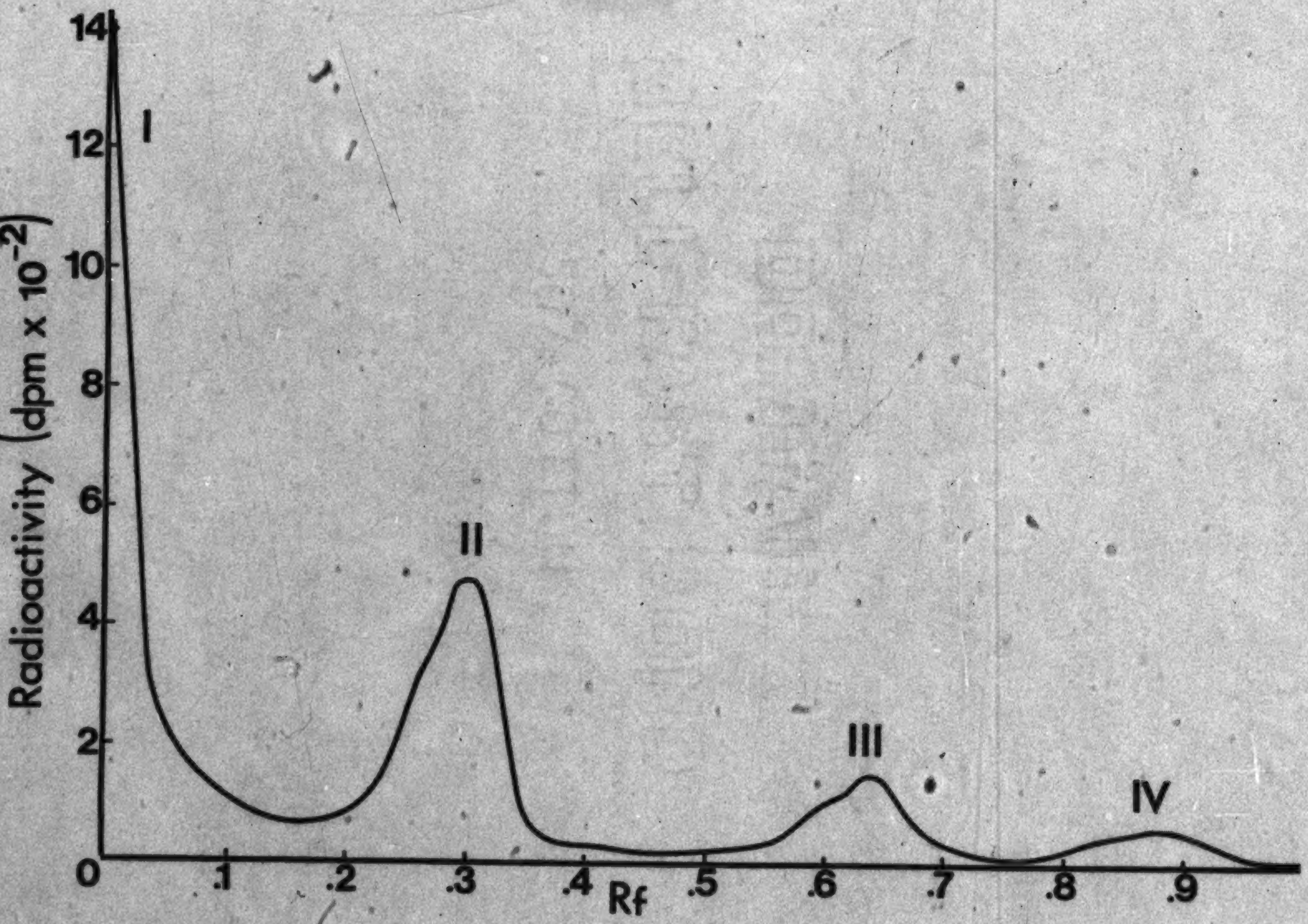
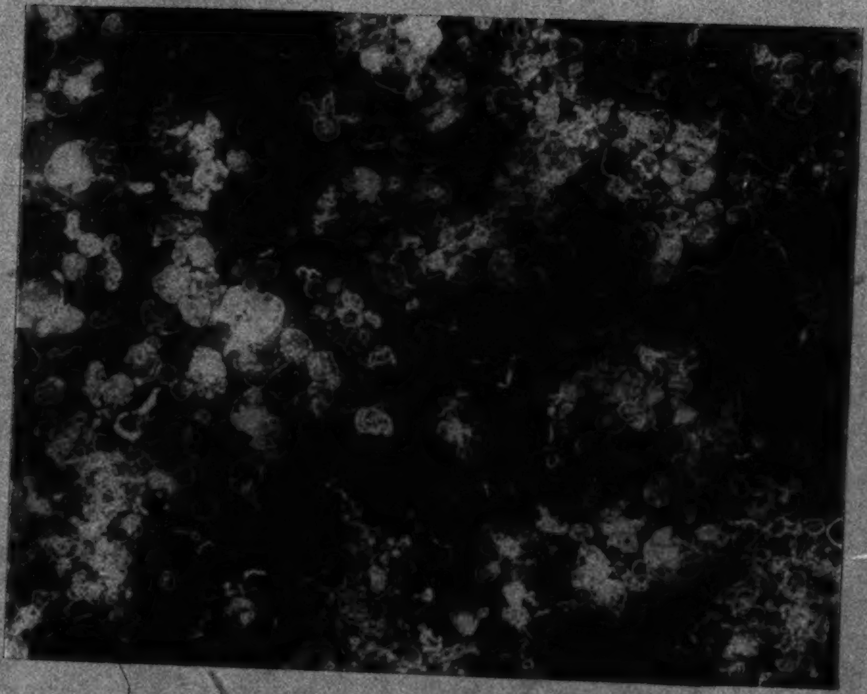

Radioactivity (dpm x 10^-2)
14
12
10
8
6
4
2
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
Rf
I
II
III
IV

PLATE I

ELECTRON PHOTOMICROGRAPH OF STATIONARY-PHASE MEMBRANE SUSPENSIONS PREPARED FROM *M. SODONENSIS*

Samples of cell membrane suspension, prepared by grinding whole cells with plastic beads in an Omni-mixer for 2 min, were negative contrast stained with 3% phosphotungstic acid.

Magnification = 23,200 X



size from approximately 0.1 to 0.3 μ in diameter. No evidence of contamination by intact cell walls or whole cells was seen.

Membrane suspensions could be stored for prolonged periods at -70°C without loss of activity. Freezing and thawing did cause loss of activity so membrane suspensions were dispensed into small amounts before freezing.

b. Grinding With Alumina. When pellets of washed stationary-phase cells were ground with 3 times their wet weight of alumina in a chilled mortar and pestle for 10 min, some cell breakage occurred, but resulting membrane suspensions had less than 50 percent of the transglycosidase activity of membrane suspensions prepared with plastic beads. Only 2.2 nmoles of MurNAc-^{14}C-pentapeptide was incorporated into peptidoglycan per hour per mg of membrane protein.

c. Lysozyme-French Press. Washed stationary-phase cells were partially digested with lysozyme and then forced through a French pressure cell. The resulting membrane suspensions were contaminated with lysozyme-insoluble cell wall material which could not be removed by differential centrifugation. These membrane suspensions showed no detectable transglycosidase activity. Unlike the membranes prepared by grinding cells with plastic beads for 10 min, these lysozyme-French press membranes did show some transglycosidase-related enzyme activities, in that small amounts of lipid precursors (Figure 3, Peak IV) were formed and production of some MurNAc-pentapeptide (Figure 3, Peak III) could also be detected.

2. Effect of Methods of Cell Breakage on Membrane Marker Enzymes. In evaluating different methods of membrane preparation, succinic dehydrogenase and NADH dehydrogenase assays were initially performed in an attempt to determine the relative activities of the resulting membrane suspensions.

Since these enzyme activities could be rapidly measured they would make convenient marker enzymes if they correlated with transglycosidase activity. These two enzymes were however much less sensitive to the method of membrane production used as evidenced by the fact that high levels of both activities were found in membrane suspensions isolated by all methods attempted, and therefore no useful correlation was found.

B. Characteristics of Transglycosidase Activity. All transglycosidase assays were done using 70 µl of stationary-phase membrane suspension.

1. Effect of pH. The effect of pH on transglycosidase activity was determined by setting up a series of normal transglycosidase assays at several pH values ranging from 7.0 to 9.0. The final concentration of Tris in the assay was kept constant at 0.125 M. Activity was greatest at a pH of 9.0 (Figure 4). Below a pH of 8.5, activity fell off sharply.

2. Effect of Temperature. The effect of temperature was determined by preparing five identical transglycosidase assays and incubating each one at a different temperature from 20 to 40°C. Transglycosidase activity was strongly temperature-sensitive (Figure 5). Activity was maximal at 30°C, which is also the optimum growth temperature for *M. sodonensis*.

3. Effect of Incubation Time. The effect of incubation time was measured by setting up a normal transglycosidase assay, scaled up 6 fold. Samples of 130 µl were withdrawn periodically during the 24 hour incubation period and boiled for 1 min to stop the reaction. After all samples had been taken they were streaked on paper strips and chromatographed in solvent A. Peptidoglycan biosynthesis proceeded rapidly for the first few hours, began to level off at 7 hours, and finally went into a decline (Figure 6). Sodium azide was incorporated into the scaled up

FIGURE 4

EFFECT OF pH ON IN VITRO TRANSGLYCOSIDASE ACTIVITY IN STATIONARY-PHASE MEMBRANE SUSPENSIONS OF M. SODONENSIS

Transglycosidase activity was measured at pH values ranging from 7.0 to 9.0, and activity was expressed as percent of total radioactivity incorporated into peptidoglycan. Details of the standard transglycosidase assay system are given in Materials and Methods.

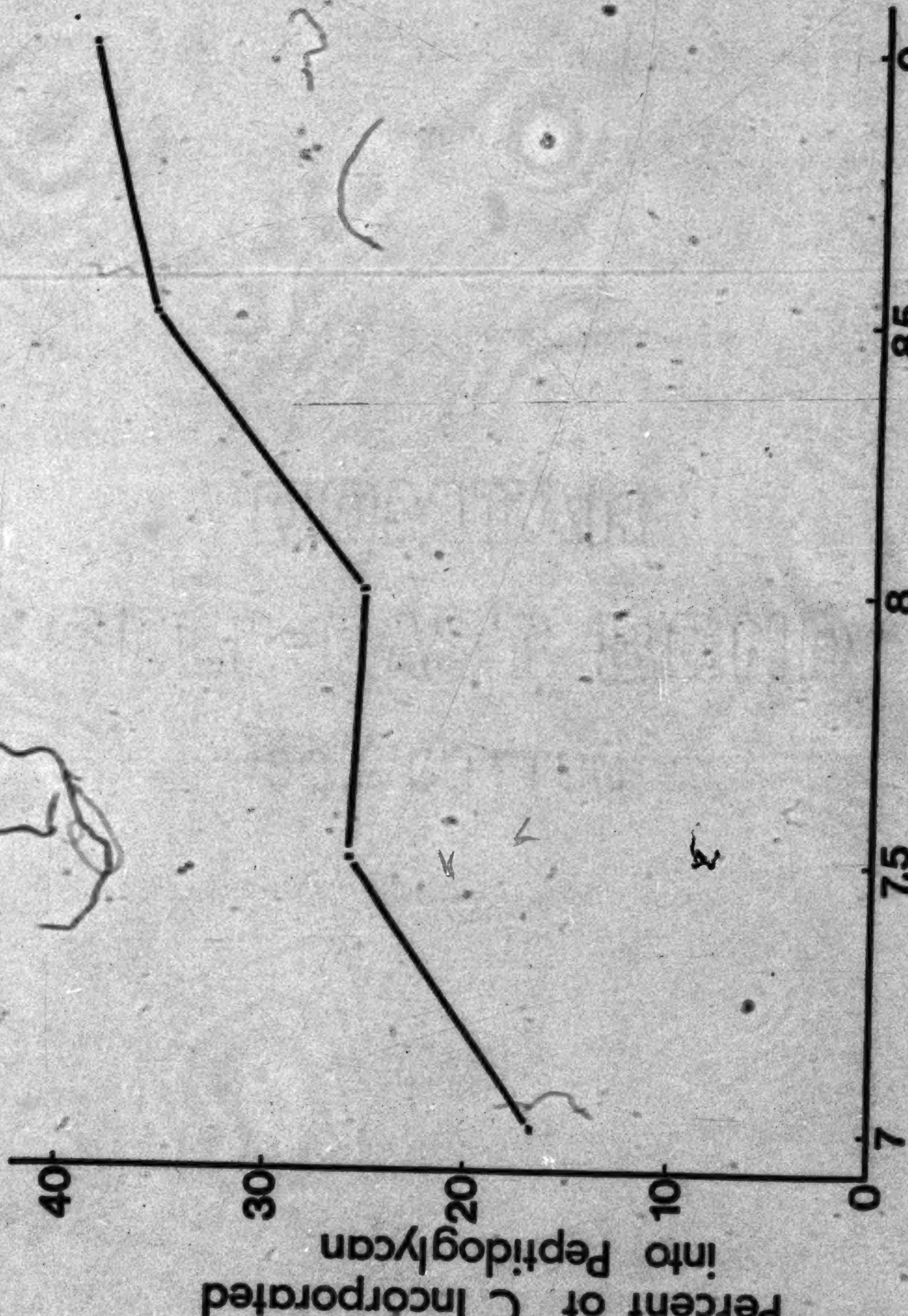

Percent of ^{14}C Incorporated into Peptidoglycan
0
10
20
30
40
pH
7
7.5
8
8.5
9

FIGURE 5

EFFECT OF INCUBATION TEMPERATURE ON *IN VITRO* TRANSGLYCOSIDASE ACTIVITY IN STATIONARY-PHASE MEMBRANE SUSPENSIONS OF *M. SODONENSIS*

Five transglycosidase assays were set up and incubated at temperatures ranging from 20°C to 40°C. Activity is expressed as percent of radioactivity incorporated into peptidoglycan.

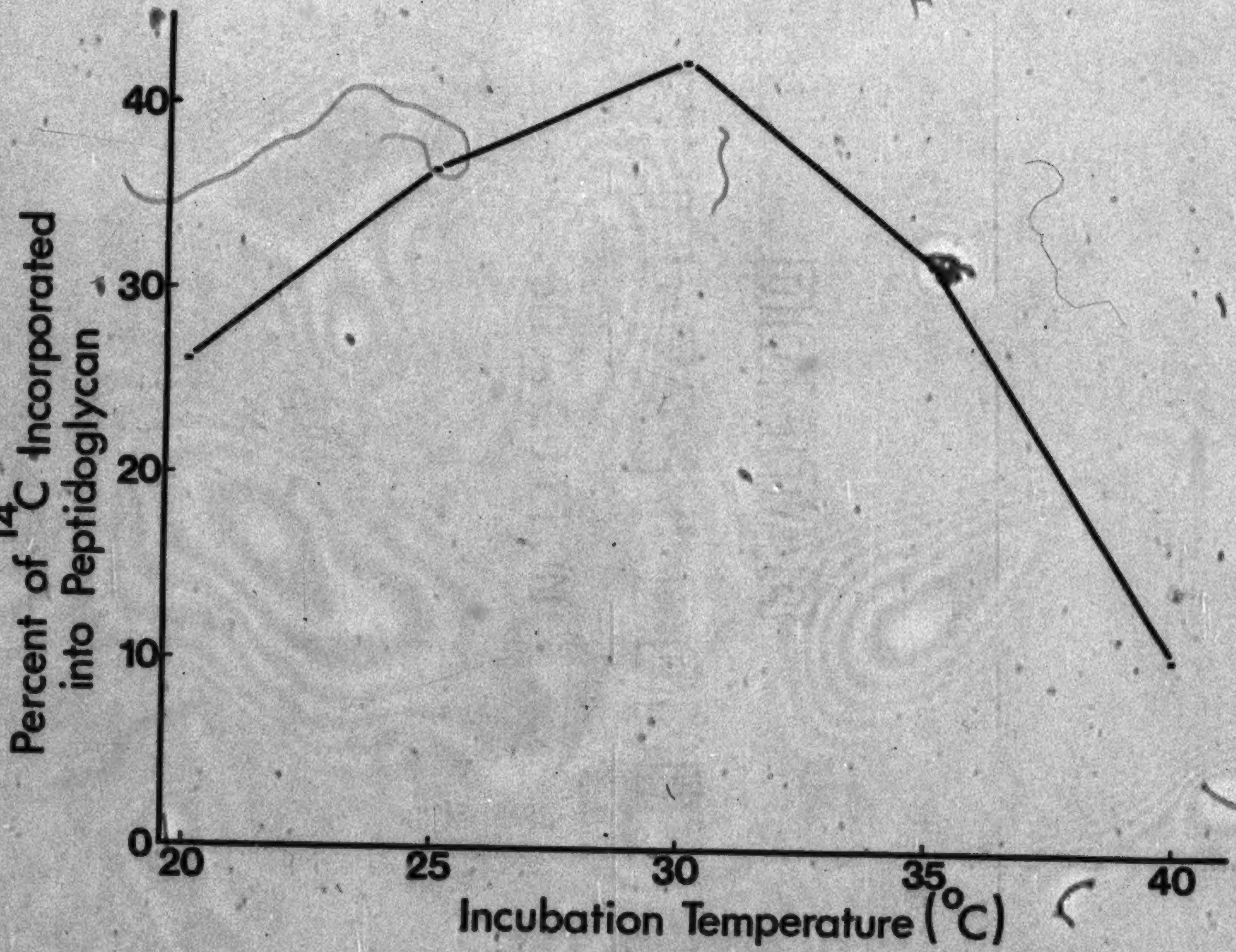
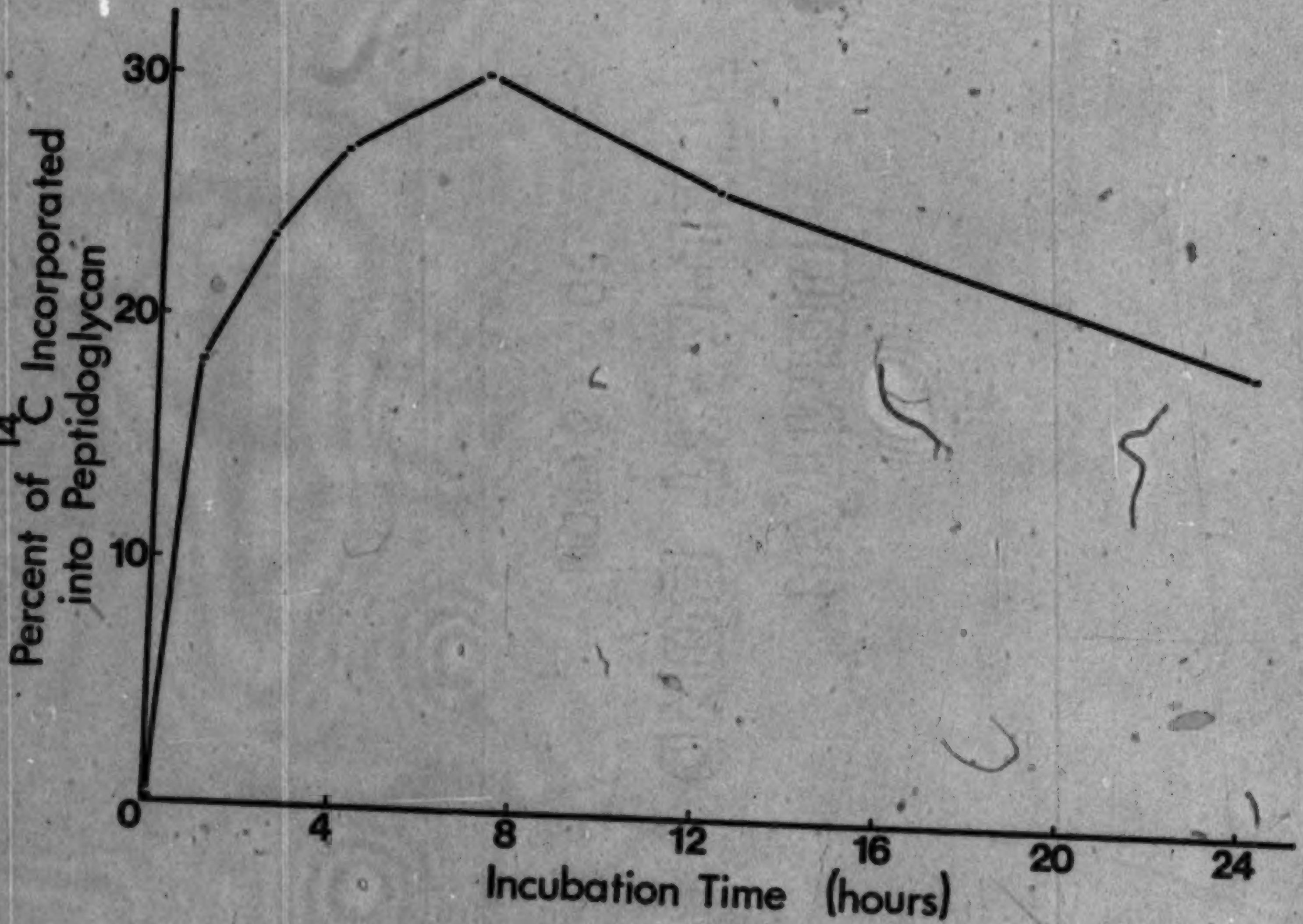

Percent of ^{14}C Incorporated into Peptidoglycan
0
10
20
30
40
20
25
30
35
40
Incubation Temperature (°C)

FIGURE 6

EFFECT OF INCUBATION TIME ON *IN VITRO* TRANSGLYCOSIDASE ACTIVITY IN STATIONARY-PHASE MEMBRANE SUSPENSIONS OF *M. SODONENSIS*

One hundred thirty µl samples of transglycosidase reaction mixture were removed at the times indicated and chromatographed in solvent A. Activity is expressed as percent of total radioactivity incorporated into peptidoglycan.


Percent of ^{14}C Incorporated into Peptidoglycan
0
10
20
30
4
8
12
16
20
24
Incubation Time (hours)

reaction mixture at a final concentration of 0.02% (w/v). Since azide would limit microbial contamination, the decline was presumed to be due to the presence of low levels of endogenous peptidoglycan degrading enzymes.

4. Effects of Mg^{2+}. Mg^{2+} is an essential component of the transglycosidase system (Figure 7). When the $MgCl_2$ concentration of the standard transglycosidase assay system was varied from zero to 125 mM, maximum transglycosidase activity occurred at 7 mM Mg^{2+}. When Mg^{2+} was omitted from the assay system, some residual activity remained, probably due to a small amount of Mg^{2+} remaining associated with the membranes throughout the washing procedure. Concentrations of Mg^{2+} in excess of 7 mM were inhibitory so that at a Mg^{2+} concentration of 100 mM, activity was almost zero.

Just as high levels of Mg^{2+} inhibited the transglycosidase enzyme assay, so the presence of Mg^{2+} during preparation of membranes decreased the activity of the resulting membrane suspensions (Figure 8). When the TME buffer used during cell breakage and subsequent washing, was supplemented with 50 mM $MgCl_2$, the resulting membrane suspensions had no transglycosidase activity. Transglycosidase activity was highest in membranes prepared without $MgCl_2$. This effect could also be demonstrated by washing active membrane preparations in TME buffer containing 50 mM $MgCl_2$. Membranes so treated could lose up to 60 percent of their transglycosidase activity but complete loss of activity was never achieved. Transglycosidase activity lost by Mg^{2+} washed membranes could not be restored by dialysis of inactivated membranes against Mg^{2+} free buffer, nor was transglycosidase activity demonstrable in the membrane washes. Addition of concentrated supernatants from Mg^{2+} washed membranes back to the inactive membranes also had no restorative effect.

FIGURE 7

EFFECT OF Mg^{2+} ON *IN VITRO* TRANSGLYCOSIDASE ACTIVITY IN STATIONARY-PHASE MEMBRANE SUSPENSIONS OF *M. SODONENSIS*

Transglycosidase activity was measured in the presence of varying concentrations of $MgCl_2$. Activity is expressed as percent of total radioactivity incorporated into peptidoglycan.

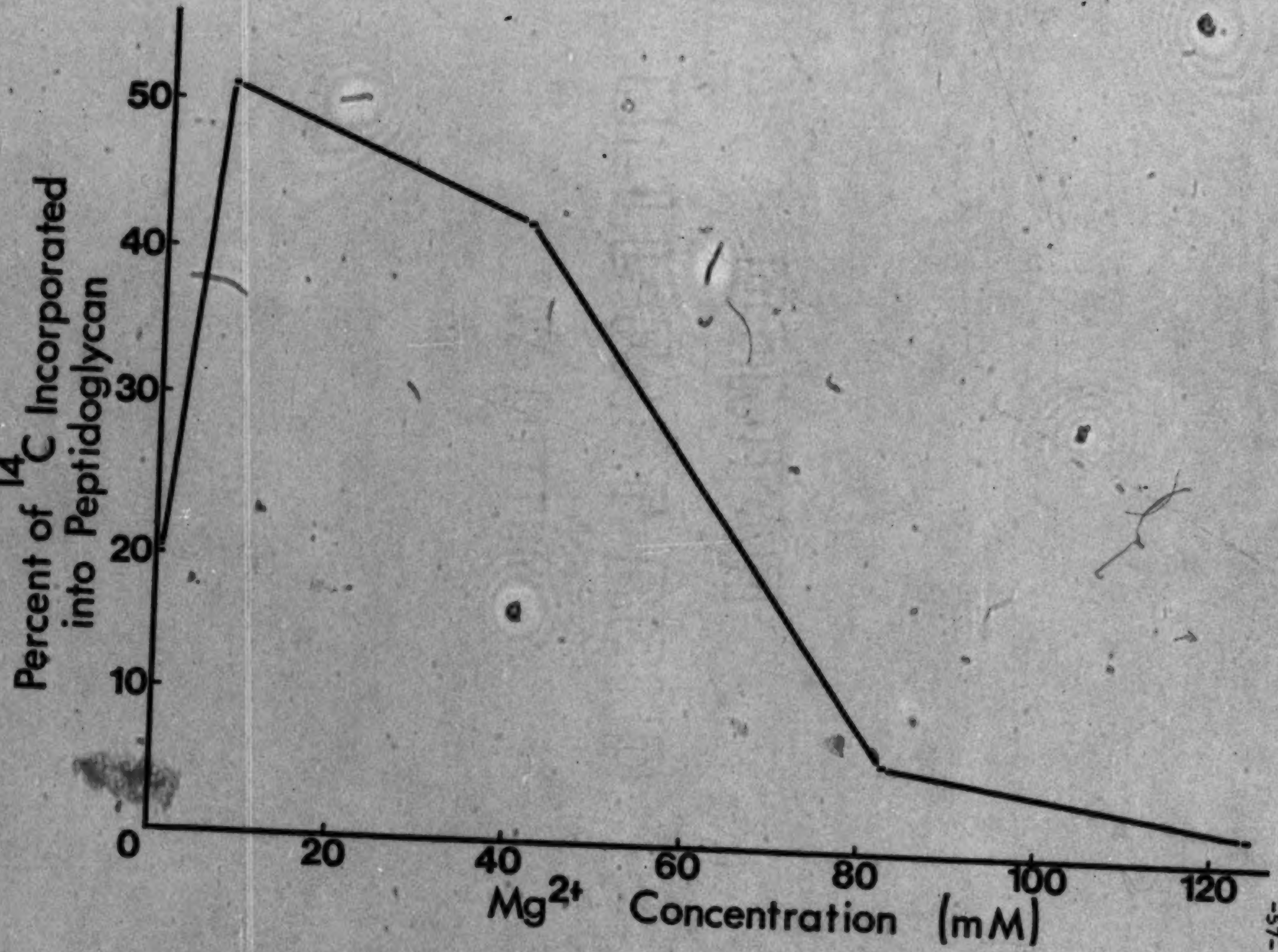
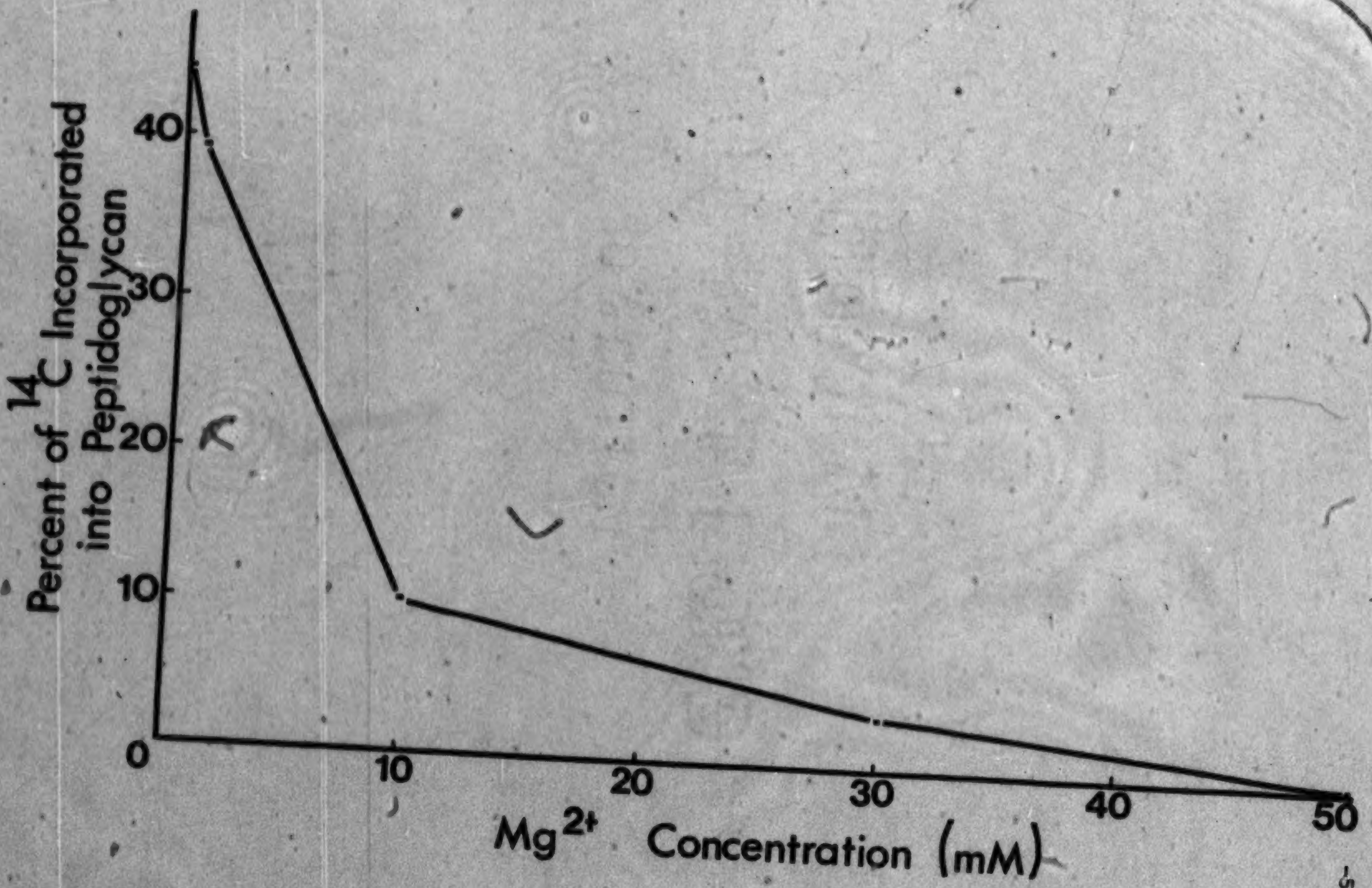

Percent of ^{14}C Incorporated into Peptidoglycan
50
40
30
20
10
0
20
40
60
80
100
120
Mg^{2+} Concentration (mM)

FIGURE 8

EFFECT OF PRESENCE OF Mg^{2+} DURING PREPARATION OF MEMBRANE SUSPENSIONS ON RESULTING TRANSGLYCOSIDASE ACTIVITY

Stationary-phase membrane suspensions, prepared using TME buffer containing varying concentrations of $MgCl_2$, were assayed for transglycosidase activity under standard assay conditions. Activity was expressed as percent of total radioactivity incorporated into peptidoglycan.


Percent of ^{14}C Incorporated into Peptidoglycan
40
30
20
10
0
10
20
30
40
50
Mg^{2+} Concentration (mM)

C. Analysis of the Transglycosidase Product.

1. Degree of Crossbridging. When cephaloridine was included in a normal transglycosidase assay at 10 μg/ml (approximately 20 times MIC) it had no effect on the amount of peptidoglycan formed or the distribution of the radioactivity on the chromatograms, which indicated that a cephaloridine-sensitive transpeptidase was not present. This, together with the observation that no free alanine was released during *in vitro* peptidoglycan synthesis is evidence that crossbridging was not occurring. Free alanine chromatographs at an Rf of 0.65 in solvent A which places it close to the Peak III material seen in Figure 3. In solvent B, however, alanine has an Rf of 0.5 compared to Peak III material which splits into two components with Rf values of 0.32 and 0.55.

2. Nature of the Glycosidic Bond Formed. The peptidoglycan produced in a standard transglycosidase assay was tested for lysozyme susceptibility, as given in Materials and Methods. The lysozyme digest was then chromatographed in solvent A and the distribution of radioactivity was compared with that of a normal transglycosidase assay (Figure 9). The complete disappearance of labelled material from the origin confirmed the transglycosidase product to be typical peptidoglycan with lysozyme sensitive N-acetylmuramyl-β 1, 4 N-acetylglucosamine linkages. The two products of lysozyme digestion (Figure 9, Peaks III and IV) were tentatively identified as disaccharide-peptide and tetrasaccharide-peptide on the basis of their chromatographic mobilities. The fact that this *in vitro* synthesized peptidoglycan is completely degraded by lysozyme differentiates it from native peptidoglycan in the mature walls of the same organism, which is at best only 50 percent solubilized by lysozyme digestion.

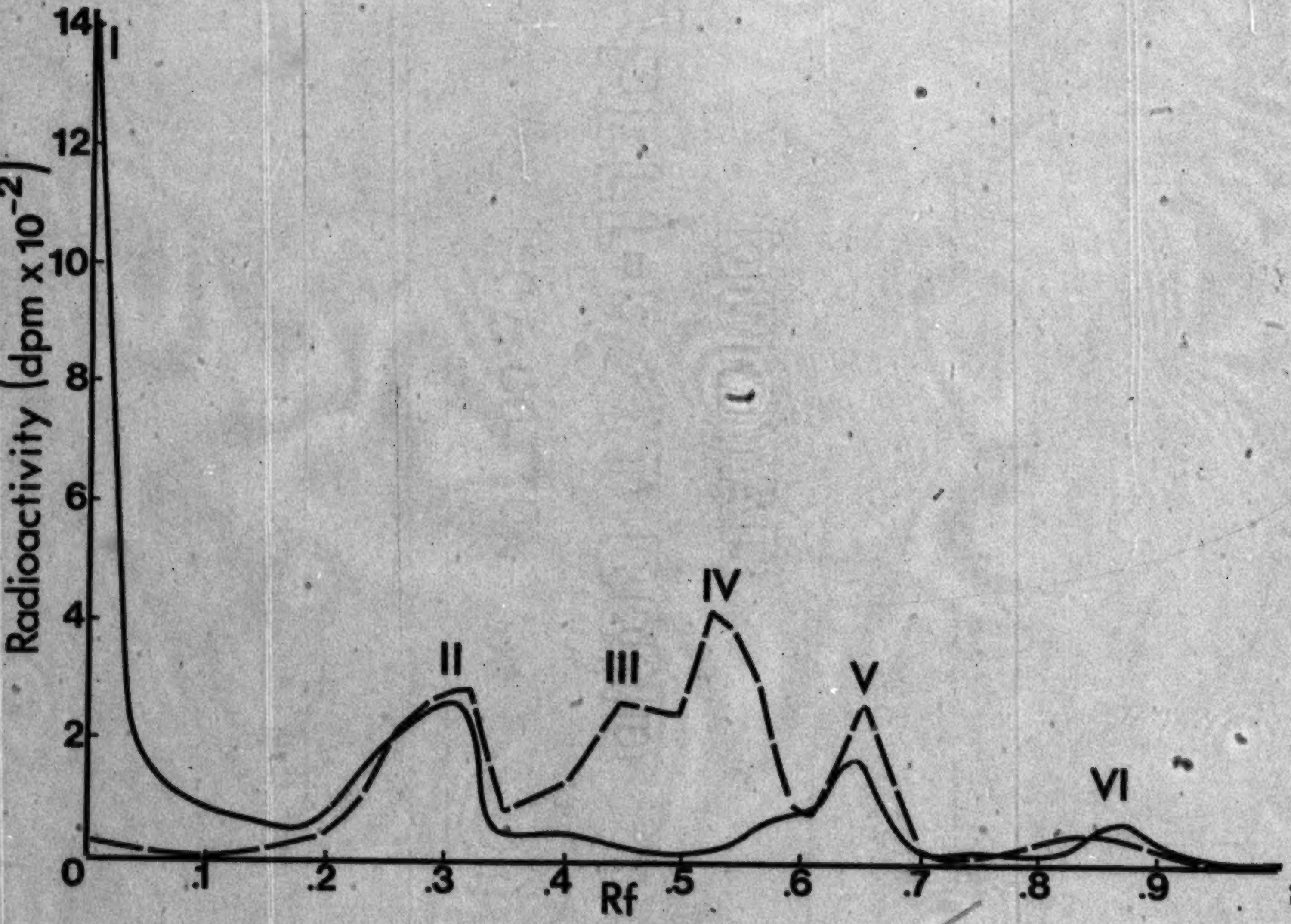
FIGURE 9

LYSOZYME DIGESTION OF PEPTIDOGLYCAN SYNTHESIZED *IN VITRO* BY STATIONARY-PHASE MEMBRANE SUSPENSIONS OF *M. SODONENSIS*

In vitro synthesized peptidoglycan was exposed to lysozyme and the digestion products were separated by paper chromatography in solvent A for 16 hours. Distribution of radioactive products was determined by cutting the strip into 1 cm sections and counting each section in a scintillation counter.

——— peptidoglycan control

— — — peptidoglycan + lysozyme


Radioactivity (dpm x 10^{-2})
14
12
10
8
6
4
2
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
Rf
I
II
III
IV
V
VI

3. Physical Characteristics

a. "Solubility" and Precipitability by Trichloroacetic Acid. The "solubility" of _in vitro_ synthesized peptidoglycan was determined by carrying out a standard transglycosidase assay using stationary-phase membranes and centrifuging the completed assay at 48,000 xg for 45 min. Peptidoglycan remaining in the 48,000 xg supernatant was referred to as "soluble" and peptidoglycan in the pellet was "insoluble". The precipitability by TCA of _in vitro_ synthesized peptidoglycan was determined by preparing two standard transglycosidase assays using stationary-phase membranes. After incubation, one reaction mixture was chromatographed in solvent A to determine the total peptidoglycan production. The other was spread evenly over a 2 cm square piece of Whatman 3MM paper. After drying, the paper was washed gently for 15 min in each of 3 changes of ice cold 5% TCA, then washed for 15 min in ethanol:ether (1:1, v/v) mixture and finally 15 min in ether. The dried square was counted for radioactivity.

Seventy seven percent of the _in vitro_ synthesized peptidoglycan was "soluble" while 61 percent could not be precipitated by TCA (Table II). Addition of cephaloridine to the transglycosidase assay system at 10 μg/ml did not alter the distribution of the various fractions.

b. Isolation and Purification of _In Vitro_ Synthesized Peptidoglycan. _In vitro_ synthesized peptidoglycan was prepared in a normal transglycosidase assay system scaled up 200 fold. One hundred mg of stationary-phase membrane protein served as the source of enzyme. The peptidoglycan produced contained 558,000 dpm which represented an incorporation of 804 nmoles of MurNAc-^{14}C-pentapeptide into peptidoglycan. The entire reaction mixture was first centrifuged at 48,000 xg for 45 min to separate "soluble" from

TABLE II

SEPARATION OF COMPONENTS OF IN VITRO SYNTHESIZED PEPTIDOGLYCAN BY CENTRIFUGATION AND TCA PRECIPITATION

Material Assayed	Radioactivity[a] Incorporated into Peptidoglycan	Percent of Total Peptidoglycan
Complete Transglycosidase Reaction Mixture (total peptidoglycan)	1470[b]	100.0
48,000 xg Supernatant ("soluble" peptidoglycan)	1135[b]	77.3
48,000 xg Pellet ("insoluble" peptidoglycan)	335[b]	22.7
Peptidoglycan Insoluble in 5% TCA	580[c]	39.0

[a] dpm

[b] Determined by paper chromatography.

[c] Determined by precipitation onto paper.

"insoluble" material. "Soluble" peptidoglycan was present in the supernatant together with residual substrate and minor amounts of degradation products. "Insoluble" peptidoglycan was that material precipitated together with the membrane fragments. It was contaminated by the radioactive lipid precursors.

The "soluble" peptidoglycan containing supernatant was lyophilized resuspended in a 2 ml volume and subjected to gel filtration on a Sephadex G-200 column (2.5 x 45 cm). Five ml fractions were collected and monitored for radioactivity (Figure 10). Some of the "soluble" peptidoglycan was excluded from the column and was therefore of very large molecular weight (Peak I), while the majority was present in a single peak (PeakII) which eluted with a Kav of 0.7. Peak III material represents residual substrate. The initial sample applied to the column had a high salt concentration, which caused shrinkage of the column and distorted the Kav of Peak II. The Peak II material was rechromatographed on Sephadex G-200 to find the true Kav and to obtain a better separation from the unreacted substrate. This peak now eluted uniformly at a Kav of 0.5. This "soluble" small molecular weight peptidoglycan was lyophilized as was the "soluble" large molecular weight material (Peak I).

"Insoluble" peptidoglycan was examined to determine whether its insolubility was due to large molecular size or to attachment to the membrane fragments. The peptidoglycan containing pellet of membrane fragments was resuspended in water to a 5 ml volume. The suspension was extracted with an equal volume of water saturated n butanol for 15 min at 4^{o}C. Centrifugation at 38,000 xg for 20 min separated the emulsion and the aqueous layer was removed. The butanol layer was discarded as it contained no

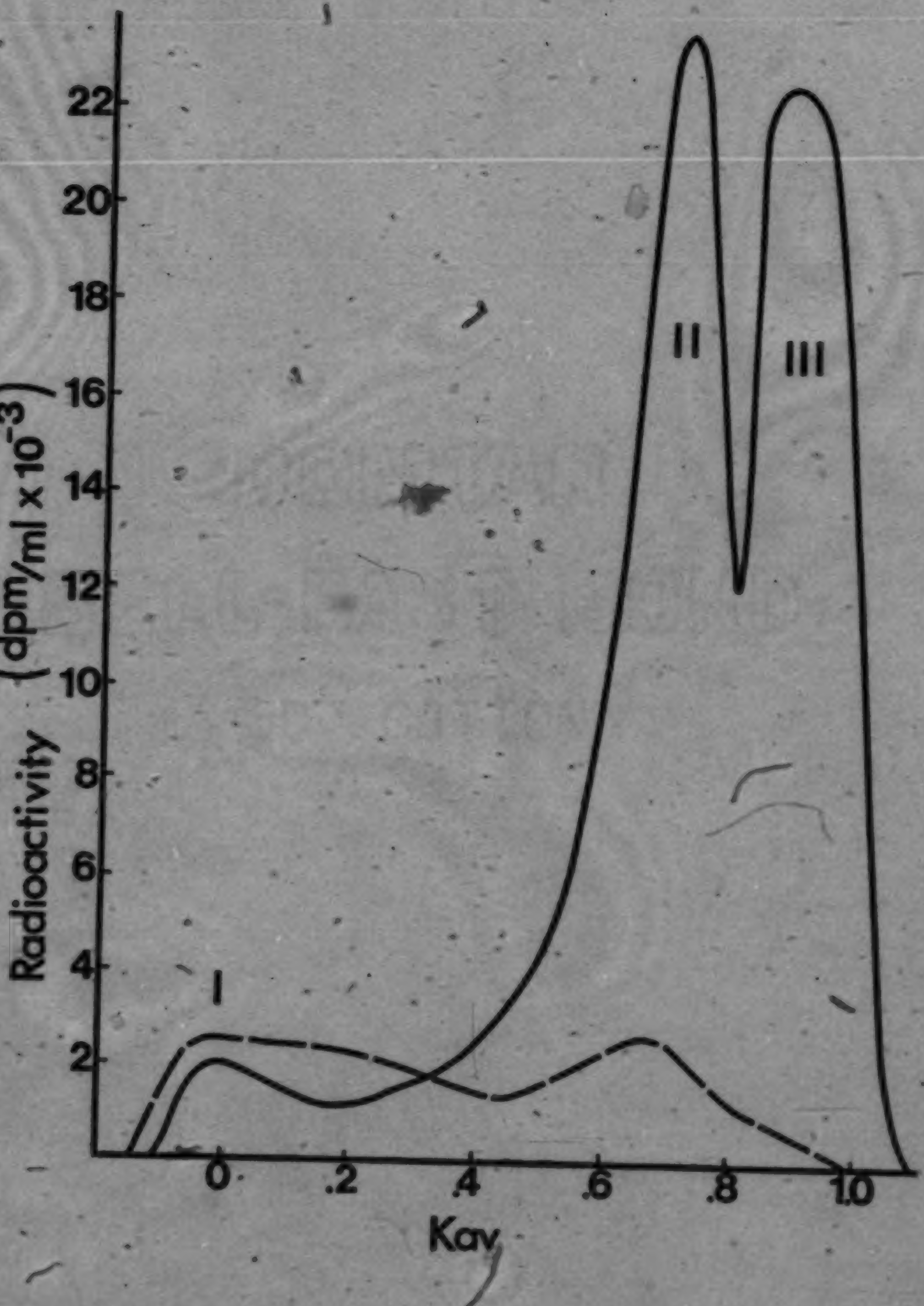
FIGURE 10

FRACTIONATION OF IN VITRO SYNTHESIZED PEPTIDOGLYCAN BY GEL FILTRATION (SEPHADEX G-200)

"Soluble" and "insoluble" *in vitro* synthesized peptidoglycan samples were applied separately to a Sephadex G 200 column (2.5 x 45 cm) and eluted with water. Five ml fractions were collected and assayed for radioactivity.

——— "soluble" peptidoglycan

— — — "insoluble" peptidoglycan


Radioactivity (dpm/ml x 10^-3)
22
20
18
16
14
12
10
8
6
4
2
I
II
III
0
.2
.4
.6
.8
1.0
Kav

radioactivity. The pellet was resuspended in 5 ml of water and the butanol extraction was repeated. Two successive butanol extractions were found to release greater than 90 percent of the "insoluble" peptidoglycan into the aqueous phase. This material could no longer be sedimented by centrifugation at 48,000 xg for 45 min. The aqueous suspension of "insoluble" peptidoglycan was applied to a Sephadex G-200 column (2.5 x 45 cm) and the effluent fractions were monitored for radioactivity (Figure 10). The elution pattern of this material was quite similar to that of the "soluble" peptidoglycan except that there was much less of the small molecular weight material and residual substrate was greatly reduced. The large and small molecular weight fractions of "insoluble" peptidoglycan were lyophilized. The yield of each of the four species of peptidoglycan is shown in Table III. The overall recovery of 788 nmoles out of an initial 804 nmoles total peptidoglycan at the start is somewhat high because the "insoluble" large molecular weight peptidoglycan is contaminated to a maximum of 20 percent with lipid intermediates which contribute to radioactivity measurements. "Soluble" small molecular weight peptidoglycan makes up the majority of the *in vitro* synthesized material. Small molecular weight material makes up about 67 percent of total peptidoglycan.

c. Hexosamine Content of *In Vitro* Synthesized Peptidoglycan. The large and small molecular weight peptidoglycan species (Figure 10, Peaks I and II) from both "soluble" and "insoluble" peptidoglycan were resuspended in water to give 35,000 to 40,000 dpm/ml. This would correspond to a hexosamine content of 50 to 60 nmoles hexosamine/ml assuming that all the peptidoglycan had been synthesized *in vitro* from radioactive substrate and that for each mole of MurNAc-^{14}C-pentapeptide incorporated one mole of GlcNAc

TABLE III

DISTRIBUTION OF FRACTIONS OF IN VITRO SYNTHESIZED PEPTIDOGLYCAN OF M. SODONENSIS

Peptidoglycan Species	Amount[a]	Percent of Total *In Vitro* Synthesized Peptidoglycan
"Soluble" large molecular weight	127	16.1
"Soluble" small molecular weight	402	51.0
"Insoluble" large molecular weight	138	17.5
"Insoluble" small molecular weight	121	15.4

[a] nmoles of MurNAc-^{14}C-pentapeptide incorporated into peptidoglycan.

was also incorporated. Using this approximation, samples were then assayed for actual total hexosamine content using the Morgen-Elson technique described earlier (Table IV). Actual hexosamine contents were higher than the assumed values which suggested that dilution of the specific activity of the nucleotide substrate had occurred and that all of the fractions of in vitro synthesized peptidoglycan contained significant amounts of unlabelled peptidoglycan material. The small molecular weight species were the least contaminated with unlabelled material. In particular, the "soluble" small molecular weight fraction, which makes up a large proportion of the in vitro synthesized peptidoglycan, contained only one half unlabelled material. This was expected since native peptidoglyean, synthesized in vivo, is neither "soluble" nor small in size. None of the evidence indicated whether labelled and unlabelled peptidoglycan was present as a mixture or whether it was actually covalently linked together.

4. Molecular Weight Determination of "Soluble" Fractions of In Vitro Synthesized Peptidoglycan.

a. Sephadex G-200 Chromatography. The size of the two "soluble" species of in vitro synthesized peptidoglycan was determined by chromatographing them on a Sephadex G-200 column which had been calibrated by chromatographing a series of dextran standards of known molecular weights. The columns were equilibrated and eluted with 0.3% NaCl to overcome any viscosity effects due to the dextran. Dextran was detected in the effluent fractions using the anthrone technique (Figure 11). Dextran T-150, molecular weight 150,000 daltons (Peak I),

TABLE IV

HEXOSAMINE CONTENT OF FRACTIONS OF IN VITRO SYNTHESIZED PEPTIDOGLYCAN OF M. SODONENSIS

Peptidoglycan Species	Hexosamine Content[a] Estimated by Radioactivity (A)	Measured by Morgen-Elson (B)	Percent of Peptidoglycan Actually Synthesized *In Vitro* (A/B x 100)
"Soluble" large molecular weight	57.8	640	9.03
"Soluble" small molecular weight	57.2	115	49.74
"Insoluble" large molecular weight	60.4	405	14.90
"Insoluble" small molecular weight	55.4	255	21.72

[a] nmoles per ml

FIGURE 11

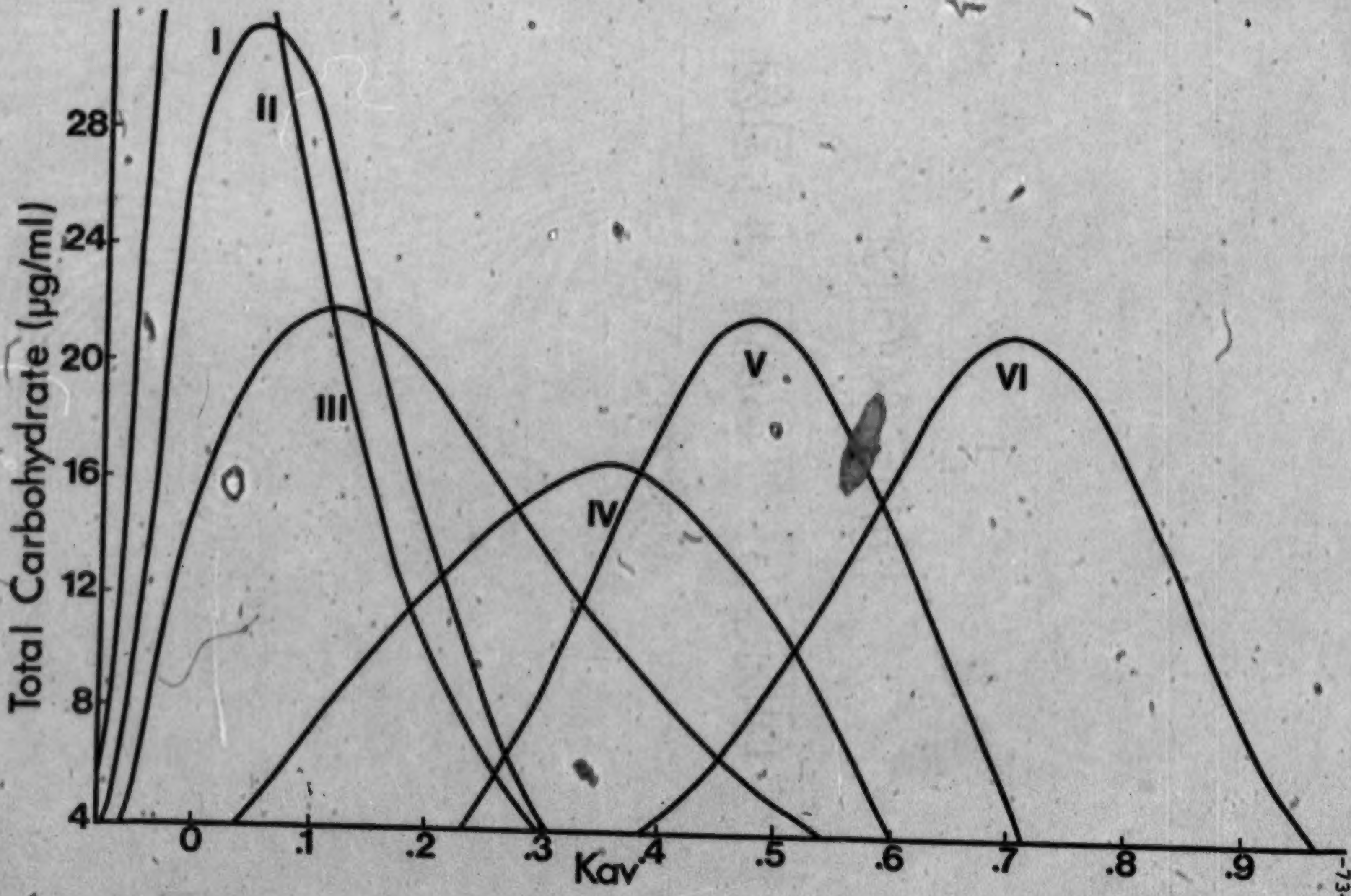
9

ESTIMATION OF PEPTIDOGLYCAN SIZE BY GEL FILTRATION

a. ELUTION PATTERN OF STANDARD DEXTRAN SAMPLES FROM SEPHADEX G-200

Two mg of each of the dextran standards were individually applied to a Sephadex G-200 column (2.5 x 45 cm). The column was equilibrated and eluted with 0.3% NaCl. Five ml fractions were collected and 0.5 ml amounts were assayed for total carbohydrate as described in Materials and Methods.

I = Dextran T-150 M. W. = 150,000 daltons
II = Dextran T-110 M. W. = 110,000 daltons
III = Dextran T-70 M. W. = 70,000 daltons
IV = Dextran T-40 M. W. = 40,000 daltons
V = Dextran T-20 M. W. = 20,000 daltons
VI = Dextran T-10 M. W. = 10,000 daltons


Total Carbohydrate (µg/ml)
4
8
12
16
20
24
28
I
II
III
IV
V
VI
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
Kav

was excluded from the column. The smaller dextrans eluted at progressively higher Kav's with Dextran T-10, molecular weight 10,000 daltons, eluting with a Kav of 0.71. The Kav of each dextran standard was then correlated with its known molecular weight (Figure 12). Kav was found to be exponentially related to molecular weight and a straight line curve was prepared. Dextrans with molecular weights in excess of 130,000 daltons would be excluded from the column. Samples of the two "soluble" peptidoglycan species, each containing 20,000 dpm of radioactivity in a 1 ml volume were then chromatographed individually on the Sephadex G-200 column (Figure 13). Effluent fractions were monitored for radioactivity. The observed Kav's were used to estimate molecular weight.

Most of the "soluble" large molecular weight peptidoglycan was excluded from the column, as expected, indicating a molecular weight in excess of 130,000 daltons. The peak had a shoulder of small material which ranged down to a Kav of 0.125, corresponding to a molecular weight of about 70,000 daltons. The "soluble" small molecular weight species eluted in a uniform peak with a Kav of 0.522 which corresponded to a molecular weight of 19,400 daltons.

The disaccharide-peptide units which make up the repeating sequence of linear peptidoglycan strands have a molecular weight of 949 daltons. The "soluble" small molecular weight peptidoglycan therefore has chains made up of 20.2 disaccharide-peptide units on the average. The "soluble" large molecular weight peptidoglycan has strands ranging in size from an average of 73.8 up to greater than 137 disaccharide-peptide units.

b. $NaBH_4$ Reduction. The number of disaccharide-peptide units making up the in vitro synthesized peptidoglycan strands could also be

FIGURE 12

ESTIMATION OF PEPTIDOGLYCAN SIZE BY GEL FILTRATION

b. DEXTRAN MOLECULAR WEIGHT CALIBRATION CURVE
FOR SEPHADEX G-200 COLUMN

I = Dextran T-10 M. W. = 10,000 daltons
II = Dextran T-20 M. W. = 20,000 daltons
III = Dextran T-40 M. W. = 40,000 daltons
IV = Dextran T-70 M. W. = 70,000 daltons
V = Dextran T-110 M. W. = 110,000 daltons
VI = Dextran T-150 M. W. = 150,000 daltons

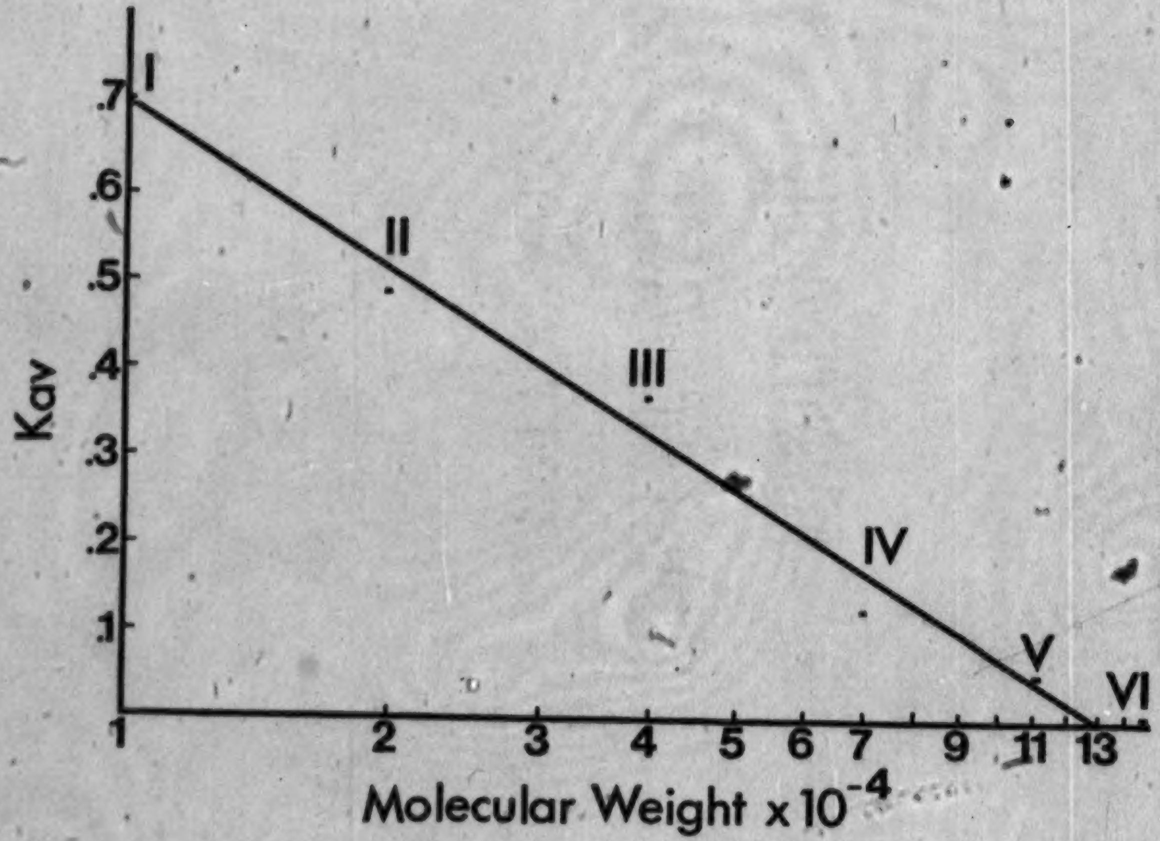

Kav
.7
.6
.5
.4
.3
.2
.1
1
2
3
4
5
6
7
9
11
13
I
II
III
IV
V
VI
Molecular Weight x 10^{-4}

FIGURE 13

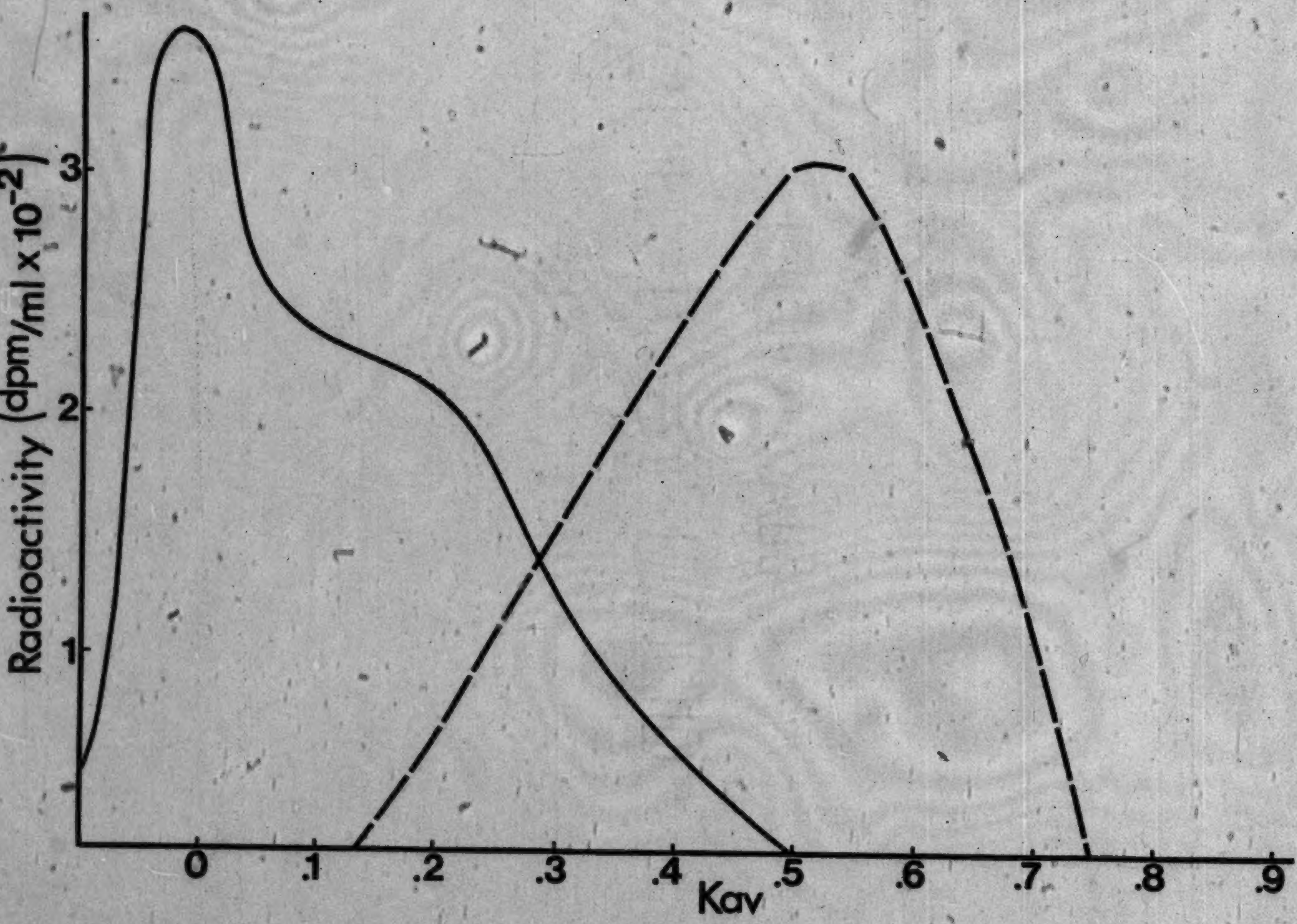
ESTIMATION OF PEPTIDOGLYCAN SIZE BY GEL FILTRATION

C. ELUTION PROFILE OF "SOLUBLE" *IN VITRO* SYNTHESI[illegible] PEPTIDOGLYCAN SPECIES FROM CALIBRATED SEPHADEX G-200 COLUMN

The large and small molecular weight species of "soluble" *in vitro* synthesized peptidoglycan were applied individually to a Sephadex G-200 column (2.5 x 45 cm). The column was equilibrated and eluted with 0.3% NaCl. Five ml fractions were collected and 0.5 ml amounts were assayed for radioactivity.

——— "soluble" large molecular weight material

— — — "soluble" small molecular weight material


Radioactivity (dpm/ml x 10^{-2})
3
2
1
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
Kav

determined by measuring the number of muramic acid residues bearing a free reducing group and comparing it to the total muramic acid content. Each peptidoglycan strand has only one free reducing group.

Samples of the "soluble" large and small molecular weight peptidoglycan containing 512 and 306 nmoles of hexosamine respectively were used. The samples were allowed to react with tritiated $NaBH_4$ (2.43 µCi/µmole) under alkaline conditions for 16 hours at 35°C. Excess reagent was destroyed by adding concentrated HCl to give a final concentration of 3N. The samples were then sealed and hydrolysed for 3 hours at 100°C; the acid was removed *in vacuo*. A standard reduction was also carried out on 1 µmole of each of glucosamine and muramic acid. The dried contents of each tube, standards and tests, were resuspended to 0.5 ml and chromatographed on Dowex 50. One ml fractions were collected and radioactivity was monitored. The elution profile of the standards is shown in Figure 14. Tritiated methyl borate, (Peak I), resulting from destruction of residual $NaBH_4$ was the first material off the column. Tritiated muramitol (Peak II) eluted just slightly behind this peak. Tritiated glucosaminol (Peak III) was retained until the second buffer system of higher pH freed it from the column. Since muramic acid is the normal reducing terminus of a peptidoglycan chain, unless it has been attacked by glycosidases, the tritiated muramitol peak was of primary importance.

The elution profile of the large and small molecular weight fractions of "soluble" peptidoglycan after reduction with tritiated $NaBH_4$ is shown in Figure 15. The level of radioactivity was very close to the background level but a peak of tritiated muramitol (Peak II) could be detected from each peptidoglycan species as a shoulder on the residual methyl

FIGURE 14

ESTIMATION OF PEPTIDOGLYCAN SIZE BY REDUCTION WITH $NaBH_4$

a. CHROMATOGRAPHIC BEHAVIOR OF ^3H MURAMITOL AND ^3H GLUCOSAMINOL STANDARDS ON DOWEX 50

Samples containing 1 μmole each of ^3H muramitol and ^3H glucosaminol were applied to a Dowex 50 column (1.0 x 20 cm). The column was eluted first with 70 ml of 0.1 M pyridine acetate buffer, pH 2.8, and then with 70 ml of 0.133 M pyridine acetate buffer, pH 3.85. One ml fractions were collected and monitored for radioactivity.

I = ^3H methyl borate
II = ^3H muramitol
III = ^3H glucosaminol

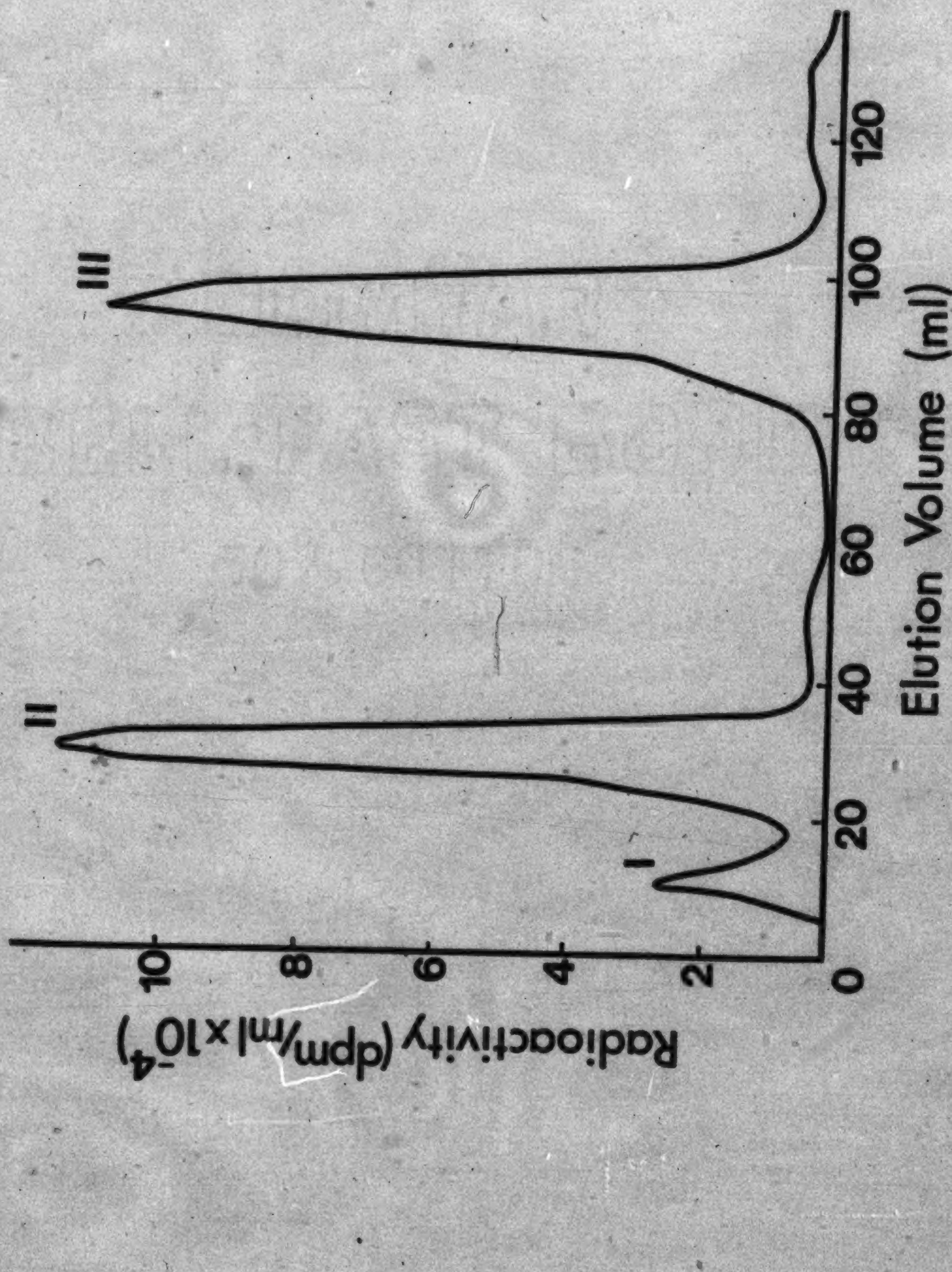

Radioactivity (dpm/ml x 10⁻⁴)
0
2
4
6
8
10
I
II
III
0
20
40
60
80
100
120
Elution Volume (ml)

FIGURE 15

ESTIMATION OF PEPTIDOGLYCAN SIZE BY REDUCTION WITH $NaBH_4$

b. ELUTION PROFILE OF "SOLUBLE" IN VITRO SYNTHESIZED PEPTIDOGLYCAN SPECIES FROM DOWEX 50

After reduction with tritiated $NaBH_4$, the peptidoglycan samples were individually resuspended in 0.5 ml of water and chromatographed on Dowex 50 columns (1.0 x 20 cm). The columns were eluted exactly the same way as for the standards. One ml fractions were collected and radioactivity was measured.

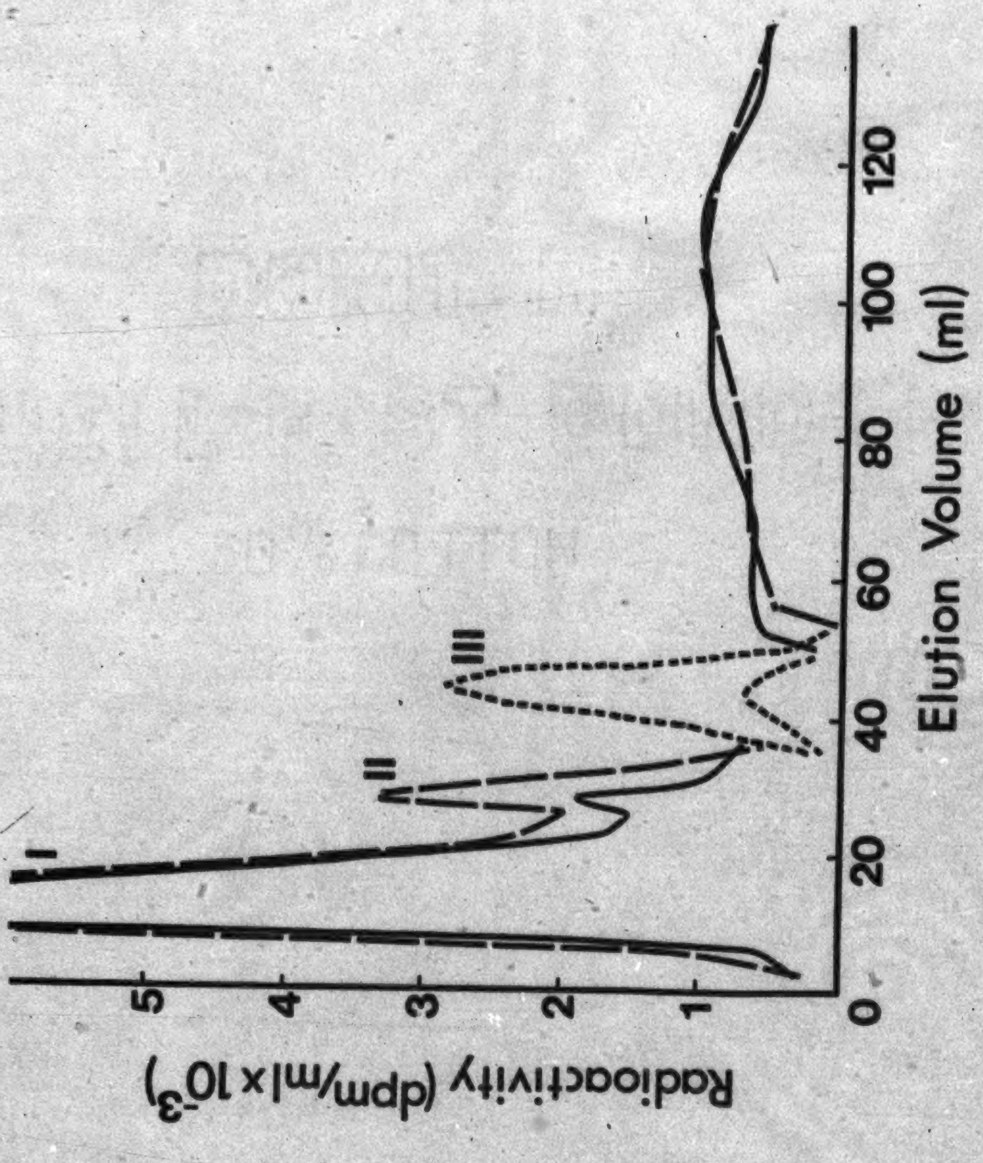
——————— "soluble" large molecular weight peptidoglycan

— — — "soluble" small molecular weight peptidoglycan

I = ^3H methyl borate

II = ^3H muramitol

III = ^{14}C pentapeptide


Radioactivity (dpm/ml x 10^{-3})
0
1
2
3
4
5
Elution Volume (ml)
20
40
60
80
100
120
I
II
III

borate (Peak I). The portion of each curve shown with a dotted line (Peak III) was where the ^{14}C labelled peptide portion of each peptidoglycan eluted. Neither peptidoglycan species showed any evidence of glucosaminol. When the total amount of radioactivity contained in each peak of muramitol from peptidoglycan was summed and compared with the amount of radioactivity contained in 1 μmole of standard muramitol, an estimate of the number of nmoles of muramic acid reduced by $NaBH_4$ could be obtained (Table V). The number of nmoles of muramic acid reduced compared to the total number of muramic acid residues gave an estimate of the chain length of the peptidoglycan. Although the limited amounts of peptidoglycan available for use in the assay may reduce the accuracy of the measurements, there was still a good agreement between the results of this and the gel filtration method. The "soluble" large molecular weight peptidoglycan had 2.4 nmoles of reducing groups on 512 nmoles of hexosamine, or 213.4 nmoles of hexosamine per reducing group. This is equivalent to 106.7 disaccharide-peptide units per strand. The "soluble" small molecular weight peptidoglycan had 7.5 nmoles of reducing groups per 306 nmoles of hexosamine, or a chain length of 20.5 disaccharide-peptide units.

III. Pyrophosphatase Activity of Stationary-Phase Membranes

Omission of UDP-GlcNAc from the standard transglycosidase assay system completely prevented peptidoglycan biosynthesis as expected. The UDP-MurNAc-^{14}C-pentapeptide did not remain unchanged but rather it was converted into a new radioactive product (Figure 16). This product chromatographed with an Rf of 0.60 in solvent A and corresponded to the

TABLE V

RESULTS OF TRITIATED $NaBH_4$ REDUCTION OF "SOLUBLE" PEPTIDOGLYCAN FRACTIONS

Material Reduced by $NaBH_4$	Radioactivity[a] in Muramitol	Reducing[b] Termini	Hexosamine Residues per Reducing Terminus	Glycan[c] Chain Length
Standard Muramic acid (1 μmole)	836,619	1,000	1	-
"Soluble" large molecular weight peptidoglycan (512 nmoles hexosamine)	2,040	2.4	213.4	106.7
"Soluble" small molecular weight peptidoglycan (306 nmoles hexosamine)	6,320	7.5	41.0	20.5

[a] dpm

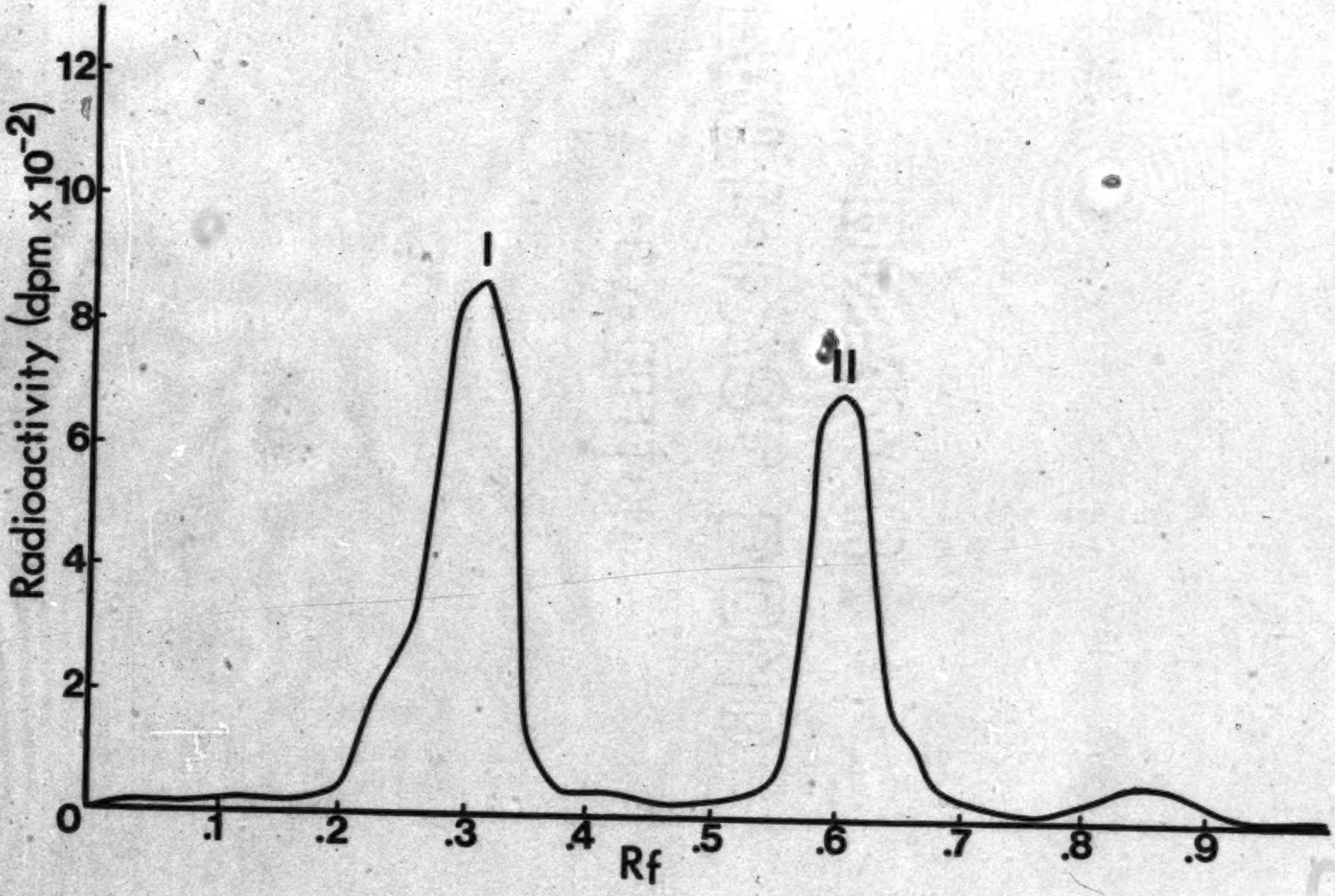
[b] nmoles

[c] number of disaccharide-peptide units per glycan strand.

FIGURE 16

PYROPHOSPHATASE ACTIVITY OF STATIONARY-PHASE MEMBRANE SUSPENSIONS OF _M_. _SODONENSIS_

One hundred forty µl of transglycosidase reaction mixture, lacking UDP-GlcNAc, was applied to a paper strip and chromatographed 16 hours in solvent A. Distribution of radioactive products was determined by cutting the strip into 1 cm sections and counting each section in a scintillation counter.


Radioactivity (dpm x 10^{-2})
0
2
4
6
8
10
12
.1
.2
.3
.4
.5
.6
.7
.8
.9
Rf
I
II

minor peak seen at this Rf value in a normal transglycosidase assay. D alanine has a Rf of 0.65 in this solvent system so the new peak had not resulted from DD carboxypeptidase activity.

A. Isolation and Purification of Pyrophosphatase Products. The unknown product was produced in larger amounts for analysis using a normal transglycosidase assay mixture scaled up 100 fold. UDP-GlcNAc was again omitted from the reaction mixture. Fifty mg of stationary-phase membrane protein served as the source of enzyme. After 3 hours incubation, the reaction was stopped by boiling, and centrifuged at 48,000 xg for 45 min to remove the membrane fragments. The supernatant was lyophilized, resuspended in 2 ml of water and applied to a Sephadex G-25 column. Fractions were monitored for radioactivity. Radioactive fractions were lyophilized, applied to paper strips and chromatographed in solvent A. Two major radioactive bands were detected on the chromatograms. One corresponded with a strongly UV-absorbing spot at an Rf of 0.3 and was identified as UDP-MurNAc-^{14}C-pentapeptide (residual substrate). The second radioactive band had no UV-absorbing characteristics and chromatographed with an Rf of 0.6. This material was eluted with water, lyophilized, and rechromatographed in solvent B. A single radioactive band was detected at an Rf of 0.55 on these chromatograms. This active material was again eluted with water, desalted on a Sephadex G-25 column and then lyophilized.

B. Identification of Pyrophosphatase Products

1. Radioactive Products. The final yield of purified material was 280,000 dpm. This corresponds to about 400 nmoles of peptide-containing product, assuming it to be of the same specific activity as the substrate. Based on this assumption, the material was resuspended into 1 ml of water

and appropriate amounts were assayed for total amino acids, soluble N-acetylhexosamines, and reducing groups as previously described. The data in Table IV shows the product to be MurNAc-^{14}C-pentapeptide. The presence of a free reducing group on muramic acid and the lack of UV-absorbing properties confirmed that the nucleotide diphosphate portion had been removed. Since the yield of MurNAc-^{14}C-pentapeptide was confirmed to be about 400 nmoles by chemical assay, no dilution of the specific activity of the nucleotide substrate had occurred.

2. Non-Radioactive Products. During the purification of MurNAc-^{14}C-pentapeptide by gel filtration, some non-radioactive, UV-absorbing material was detected. Chromatography of MurNAc-^{14}C-pentapeptide in solvent A also separated out UV-absorbing non-radioactive contaminants. This UV-absorbing material was eluted from the chromatograms, combined with the fractions from gel filtration and lyophilized. Chromatography of this material in solvents A and B indicated that two separate compounds, UMP and uridine were present. The identity of these materials was confirmed by co-chromatography with known standards. The presence of uridine is explicable as a product of degradation of UMP by the phosphomonoesterase which _M. sodonensis_ elaborates (Berry and Campbell, 1970; Mills and Campbell, 1974).

C. _Characteristics of Pyrophosphatase Activity_. All pyrophosphatase assays used 70 µl of stationary-phase membrane suspension as a source of enzyme.

1. Effects of Mg^{2+}. The requirement of pyrophosphatase for Mg^{2+} was tested by incorporating $MgCl_2$ into the standard pyrophosphatase assay

TABLE VI

COMPOSITION OF PRODUCT OF PYROPHOSPHATASE ACTIVITY ON UDP-MurNAc-^{14}C-pentapeptide

Component	Amount[a]	Molar Ratio
Glutamic acid	397	1.00
Alanine	1330	3.30
Lysine	423	1.06
Soluble-N-acetyl-hexosamine	370	0.93
Reducing groups	369	0.93

[a] nmoles per ml

system at final concentrations ranging from zero to 130 mM (Figure 17). Like the transglycosidase enzyme system, pyrophosphatase had a definite requirement for Mg^{2+} with maximum activity occurring at concentrations ranging from 42 to 84 mM. No extreme sensitivity to high Mg^{2+} concentrations was detected.

The effect of Mg^{2+}, when present during preparation of membrane suspensions, was tested by incorporating $MgCl_2$ into the TME buffer at concentrations varying from zero to 50 mM (Figure 18). The inactivating effect of Mg^{2+} on transglycosidase activity when present during membrane preparation was also seen to a lesser extent for pyrophosphatase activity where a 55 percent loss of activity was observed at 50 mM Mg^{2+}.

2. Effect of pH. The effect of pH on pyrophosphatase activity was determined by varying the pH of the Tris buffer in the standard assay system from 7.0 to 9.0 (Figure 19). The enzyme was found to be most active in the pH range of 8.0 to 9.0 with maximum activity observed at a pH of 8.5. This unusually high pH optimum is characteristic of both transglycosidase and pyrophosphatase activity.

3. Effect of Temperature. The effect of temperature on pyrophosphatase activity was tested using standard reaction mixtures incubated at temperatures in the 20 to 40°C range (Figure 20). Pyrophosphatase activity was less sensitive to temperature changes than transglycosidase activity. Optimum activity was at 25°C rather than 30°C.

4. Effect of Uridine and Related Nucleotides. Several end products or related compounds were tested for their effect on pyrophosphatase activity (Table VII). Twenty five nmoles of uridine, UMP, UDP, and UDP-glucose was added to a normal pyrophosphatase assay system and mixed well

FIGURE 17

EFFECT OF Mg^{2+} ON PYROPHOSPHATASE ACTIVITY OF STATIONARY-PHASE MEMBRANE SUSPENSIONS OF _M. SODONENSIS_

Pyrophosphatase activity was measured in assay systems containing varying concentrations of $MgCl_2$. Activity was expressed as the percent of total radioactivity incorporated into MurNAc-peptide (Rf = 0.6). Details of the standard pyrophophatase assay system used are given in Materials and Methods.

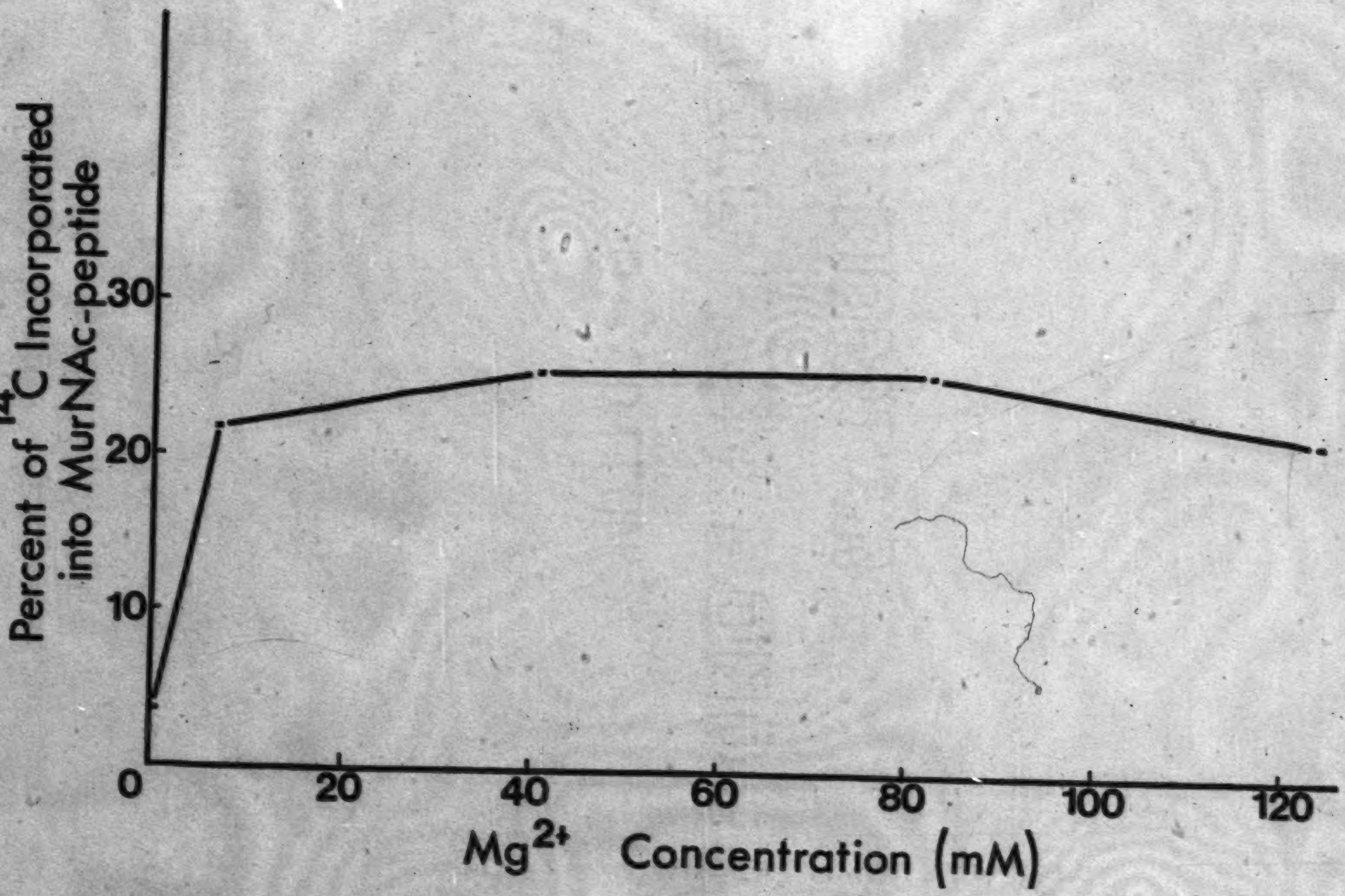

Percent of 14C Incorporated into MurNAc-peptide
0
10
20
30
0
20
40
60
80
100
120
Mg2+ Concentration (mM)

FIGURE 18

EFFECT OF PRESENCE OF Mg^{2+} DURING PREPARATION OF MEMBRANE SUSPENSIONS ON RESULTING PYROPHOSPHATASE ACTIVITY

Stationary-phase membrane suspensions, prepared using TME buffer containing varying concentrations of $MgCl_2$, were assayed for pyrophosphatase activity under standard assay conditions. Activity was expressed as percent of total radioactivity incorporated into MurNAc-peptide.

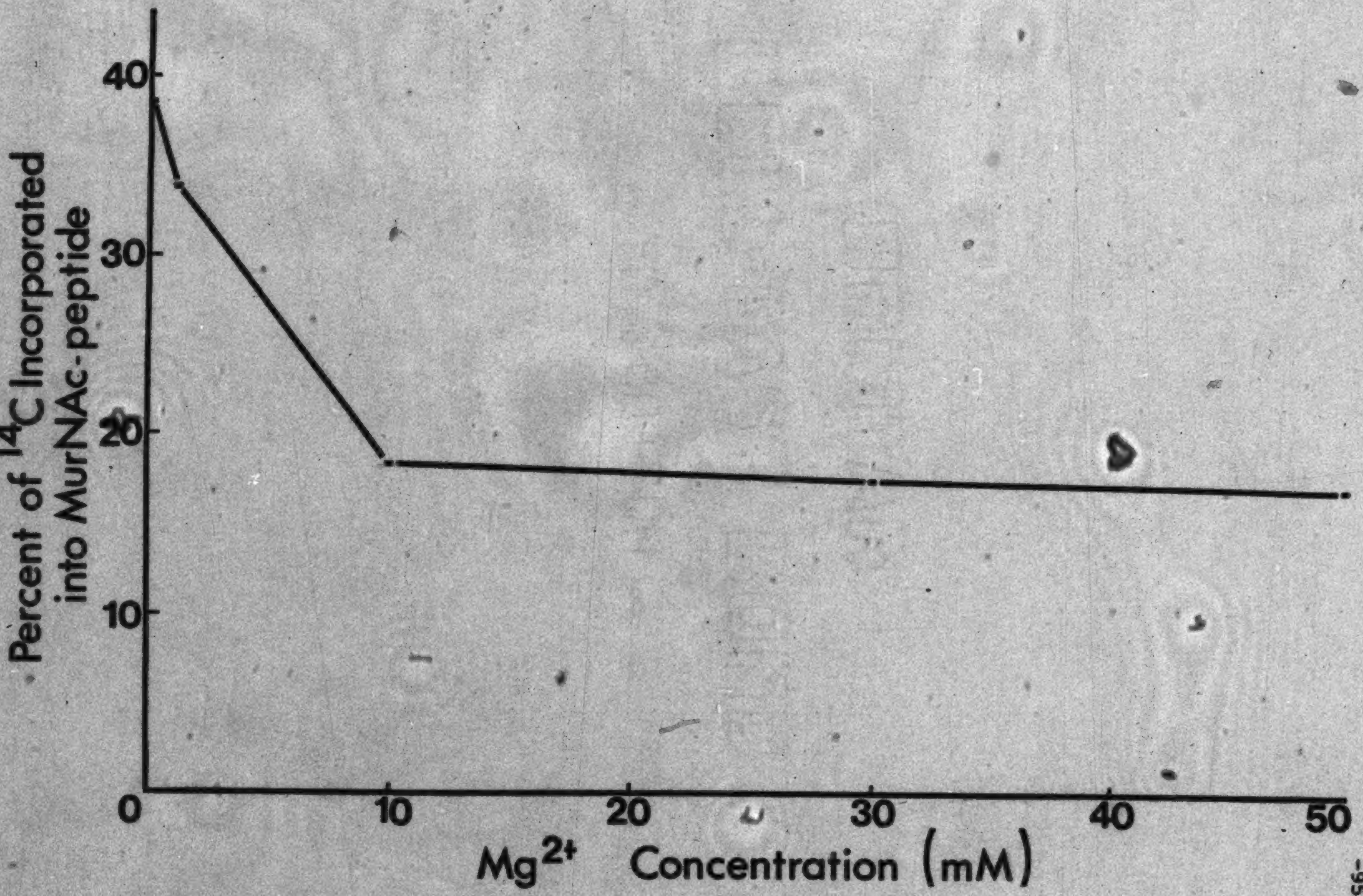

Percent of ^{14}C Incorporated into MurNAc-peptide
40
30
20
10
0
10
20
30
40
50
Mg^{2+} Concentration (mM)

FIGURE 19

EFFECT OF pH ON PYROPHOSPHATASE ACTIVITY OF STATIONARY-PHASE MEMBRANE SUSPENSIONS OF M. SODONENSIS

Pyrophosphatase activity was measured at pH values ranging from 7.0 to 9.0, and activity was expressed as percent of total radioactivity incorporated into MurNAc-peptide.

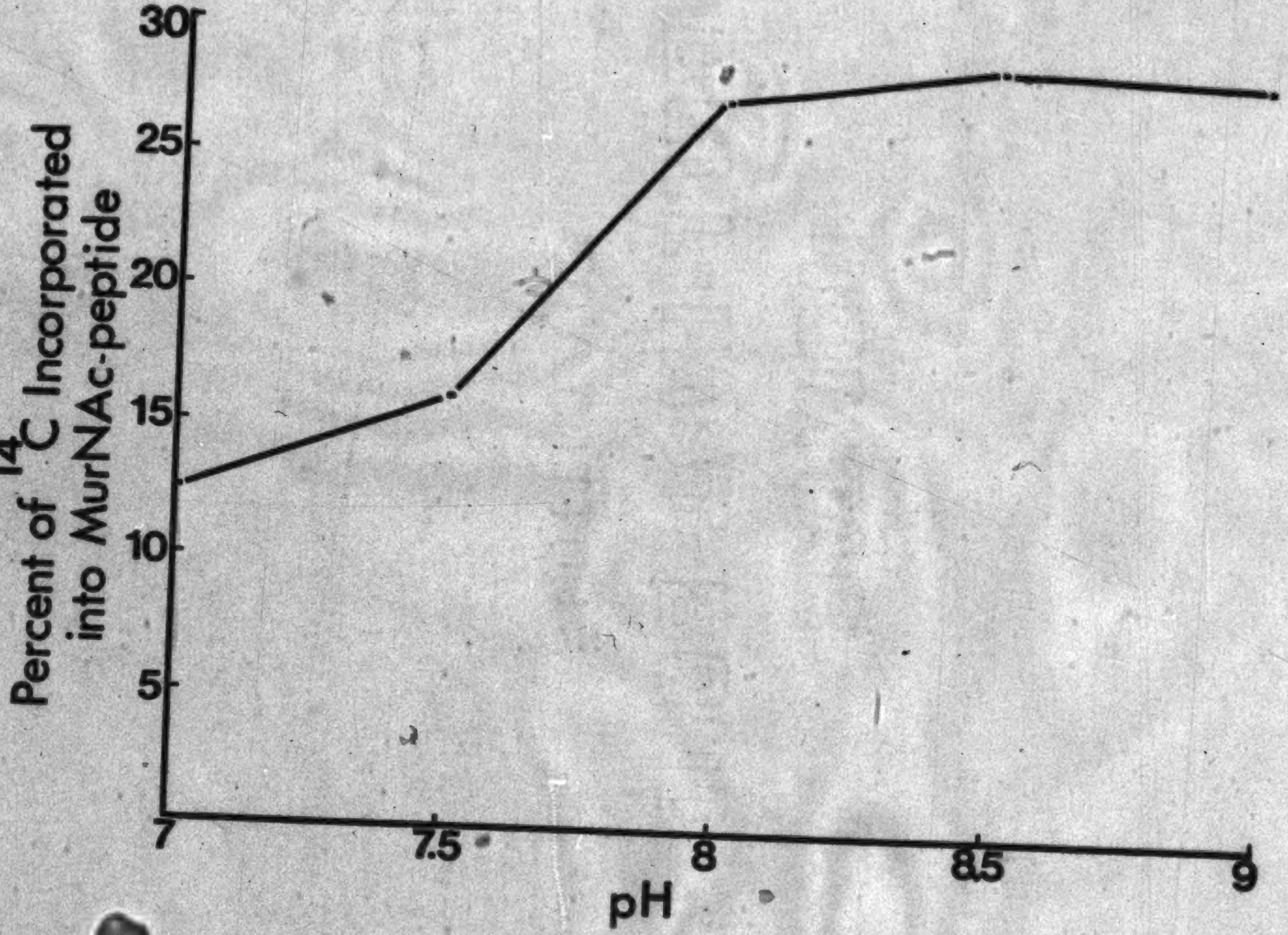

Percent of ^{14}C Incorporated into MurNAc-peptide
30
25
20
15
10
5
7
7.5
8
8.5
9
pH

FIGURE 20

EFFECT OF INCUBATION TEMPERATURE ON PYROPHOSPHATASE ACTIVITY OF STATIONARY-PHASE MEMBRANE SUSPENSIONS OF *M. SODONENSIS*

Five pyrophosphatase assays were set up and incubated at temperatures between 20 and 40°C. Activity was expressed as percent of total radioactivity incorporated into MurNAc-peptide.

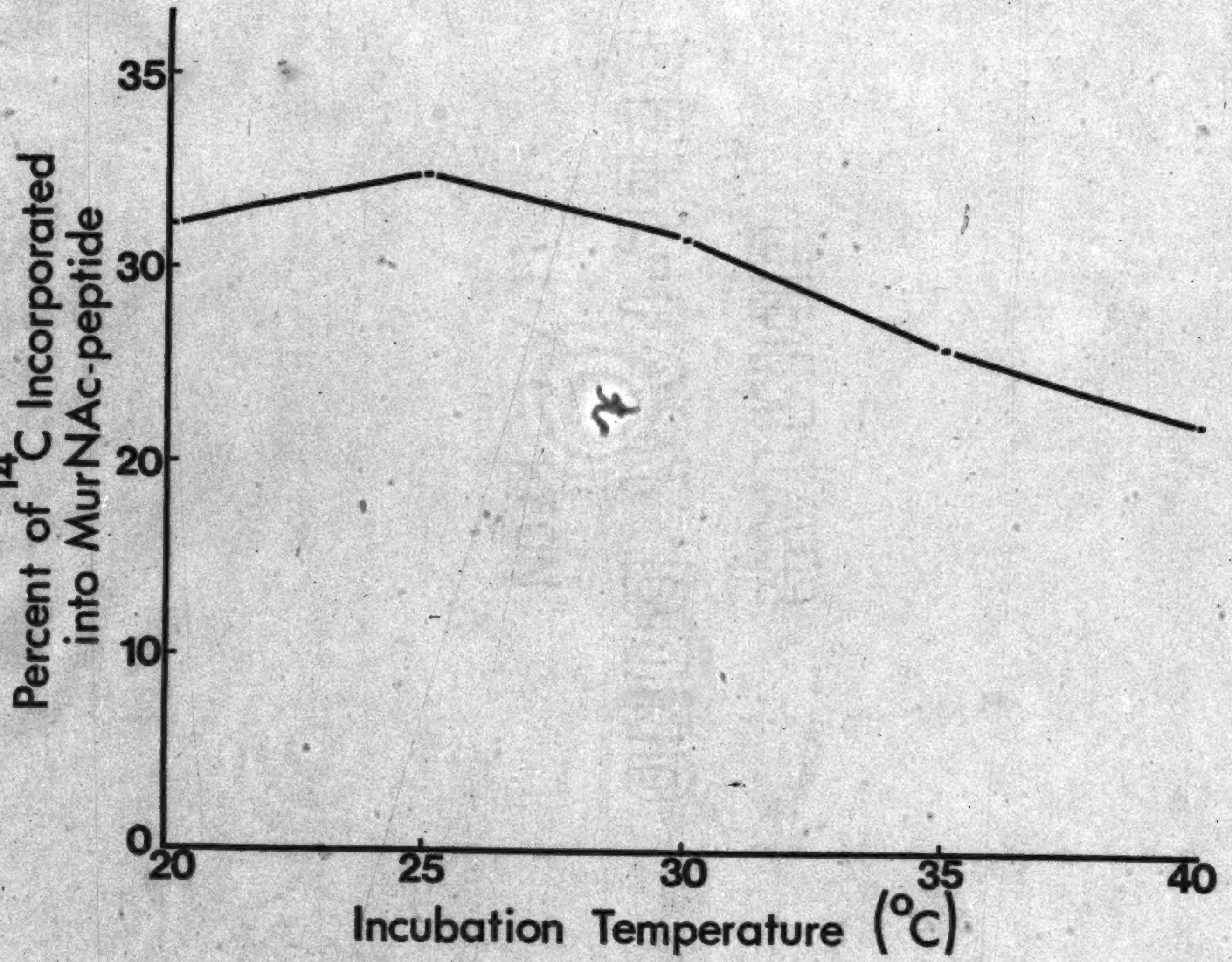

Percent of ^{14}C Incorporated into MurNAc-peptide
35
30
20
10
0
20
25
30
35
40
Incubation Temperature (°C)

TABLE VII

EFFECT OF URIDINE AND RELATED NUCLEOTIDES ON PYROPHOSPHATASE ACTIVITY IN MEMBRANE SUSPENSIONS OF *M. SODONENSIS*

Additions (25 nmoles)	MurNAc-^{14}C-pentapeptide[a] produced	% Inhibition
None	37.2	0
Uridine	39.8	0
UMP	11.4	69.4
UDP	34.2	8.1
UDP-glucose	23.1	37.9

[a] Percent of total radioactivity incorporated into MurNAc-^{14}C-pentapeptide.

before the suspension was added. Each assay was incubated for 3 hours at 25°C and chromatographed in solvent A. UMP, the proposed end product was the most effective inhibitor tested, while uridine and UDP had almost no effect. UDP-glucose was moderately effective as an inhibitor.

5. Effect of Presence of UDP-GlcNAc. The proposal that pyrophosphatase and transglycosidase activities are competing systems, regulated by the availability of UDP-GlcNAc, was tested by measuring the effect of varying concentrations of UDP-GlcNAc on the resultant distribution of the two activities. The results in Figure 21 show that pyrophosphatase activity varies inversely with the amount of UDP-GlcNAc present. UDP-GlcNAc was incorporated into the normal pyrophosphatase assay system in amounts varying from 0 to 20 nmoles. Each reaction mixture was chromatographed, and the amount of radioactivity in both peptidoglycan and MurNAc-^{14}C-pentapeptide was measured. When the ratio of UDP-GlcNAc/UDP-MurNAc-^{14}C-pentapeptide equalled or exceeded 1, transglycosidase activity was maximal and pyrophosphatase activity was minimal.

IV. Solubilization of Transglycosidase and Pyrophosphatase Activities

Three methods commonly used to solubilize membrane or cell wall associated enzymes were used in an attempt to release transglycosidase and pyrophosphatase in an active form. Details of each procedure are given in Materials and Methods. No completely satisfactory method of

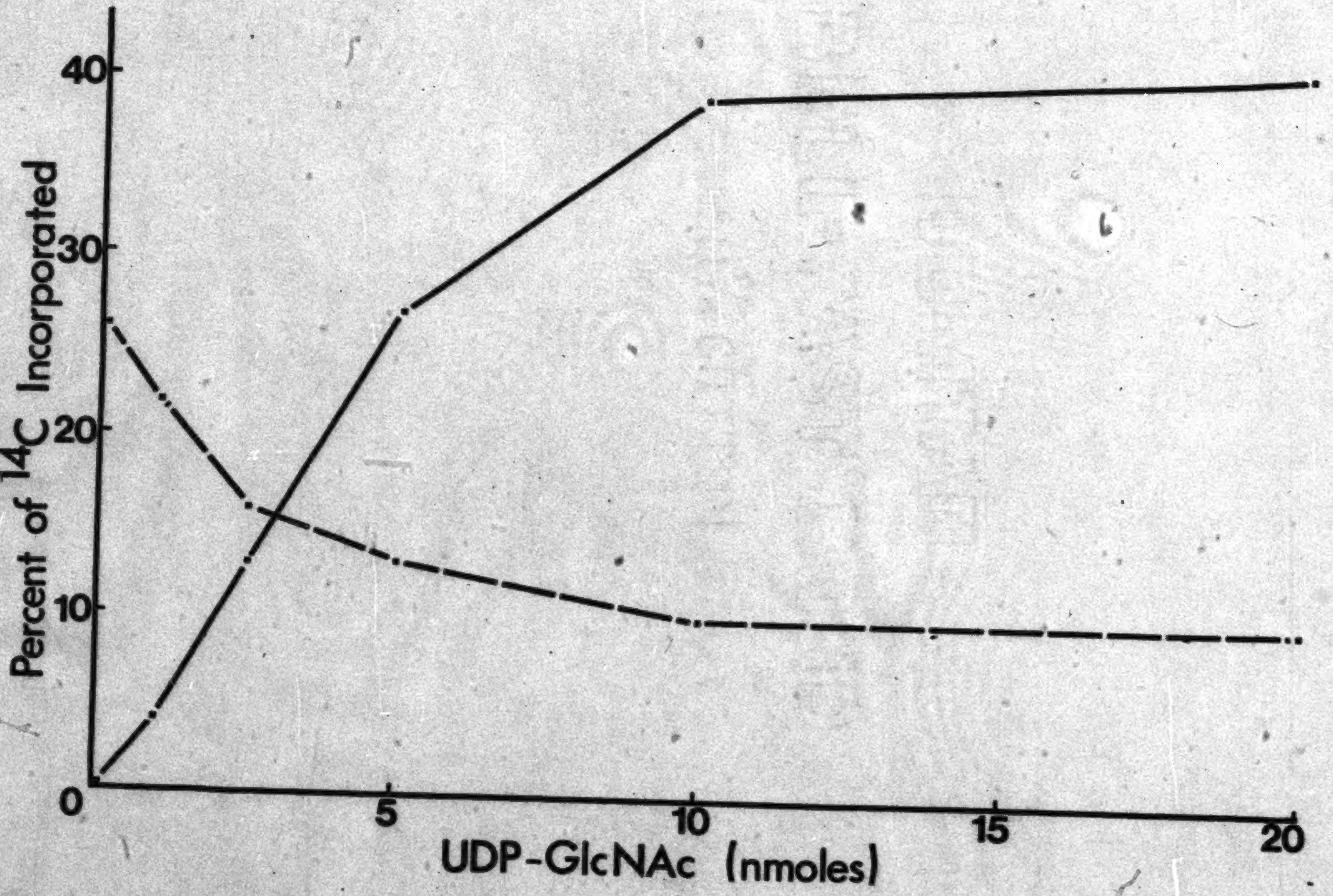
FIGURE 21

EFFECT OF UDP-GlcNAc ON DISTRIBUTION OF PYROPHOSPHATASE AND TRANSGLYCOSIDASE ACTIVITIES IN STATIONARY-PHASE MEMBRANE SUSPENSIONS OF *M. SODONENSIS*

Increasing amounts of UDP-GlcNAc were added back to a normal pyrophosphatase assay system (containing 12 nmoles of UDP-MurNAc-^{14}C-pentapeptide). Both pyrophosphatase and transglycosidase activities were measured for each amount of UDP-GlcNAc and expressed as percent of total radioactivity incorporated into MurNAc-peptide and peptidoglycan respectively.

———— transglycosidase

— — — pyrophosphatase


Percent of ^{14}C Incorporated
0
10
20
30
40
5
10
15
20
UDP-GlcNAc (nmoles)

solubilization was found (Table VIII). Triton X-100 treatment caused extensive solubilization of membrane material but only about 20 percent of both of the activities was found free in the solution. The remaining 80 percent of both activities was destroyed since the insoluble membrane residue was almost inactive. Butanol extraction also caused extensive solubilization of the membrane material but little or no activity survived the extraction. LiCl extraction, designed primarily for freeing cell wall-bound enzymes, caused no apparent change in the amount or consistency of the membrane fragments upon centrifugation, but effectively destroyed both activities.

V. Amidase Activity of Exponential-Phase Membrane Suspensions

Membrane suspensions were prepared from cells in both the stationary and the mid-exponential phases of growth. The cells were broken by grinding with plastic beads in an Omni-mixer for 2 min. The resulting membrane suspensions were compared for transglycosidase activity using the standard assay system described earlier. Unexpectedly, membranes prepared from cells in the exponential-phase of growth had much lower levels (about 1/3) of transglycosidase activity than membranes prepared from cells in the stationary-phase of growth (Figure 22). As well as residual substrate, the chromatograms from reaction mixtures containing exponential-phase membrane suspensions showed a large peak of radioactivity at an Rf of 0.65 (Peak III). This peak was almost non-existent in chromatograms from reaction mixtures containing stationary-phase membrane suspensions. Although this material co-chromatographed with alanine in solvent A, in solvent B alanine had an Rf value of 0.5 compared to the unknown peak with an Rf of

TABLE VIII

SOLUBILIZATION OF TRANSGLYCOSIDASE AND PYROPHOSPHATASE ACTIVITIES FROM STATIONARY-PHASE SUSPENSIONS OF *M. SODONENSIS*

Solubilization Technique	Transglycosidase		Pyrophosphatase	
	percent solubilized	percent remaining bound	percent solubilized	percent remaining bound
Triton X-100	21.7	1.5	19.8	2.3
Butanol Extraction	3.7	1.4	3.0	3.2
LiCl Extraction	0	0	0	0

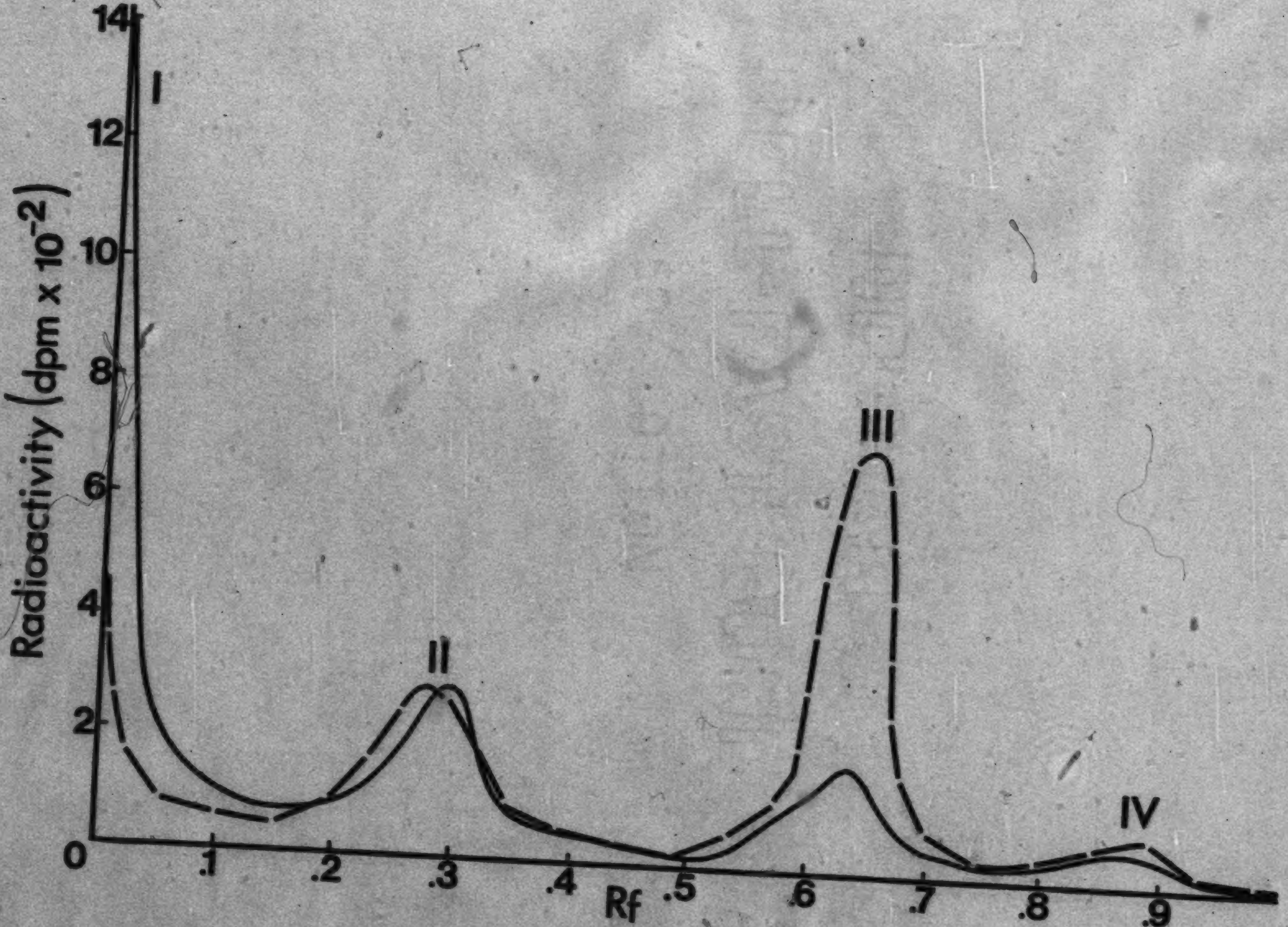
FIGURE 22

TRANSGLYCOSIDASE ACTIVITY *IN VITRO* OF EXPONENTIAL AND STATIONARY-PHASE MEMBRANE SUSPENSIONS OF *M. SODONENSIS*

Two transglycosidase reaction mixtures, one containing log membrane suspension, and the other stationary membrane suspension, were streaked on paper strips and chromatographed 16 hours in solvent A. Distribution of radioactive products was determined by cutting the strips into 1 cm sections and counting each section in a scintillation counter.

——————— Stationary-phase Membrane Suspension

— — — Exponential-phase Membrane Suspension


Radioactivity (dpm x 10^{-2})
0
2
4
6
8
10
12
14
I
II
III
IV
.1
.2
.3
.4
Rf
.5
.6
.7
.8
.9

0.32. Similarly, this new material was distinguished from MurNAc-^{14}C-pentapeptide with an Rf of 0.55 in solvent B. The new peak therefore did not result from transpeptidase or pyrophosphatase activity. In an effort to demonstrate transpeptidase activity, a special transpeptidase reaction mixture was used to assay exponential-phase and stationary-phase membrane suspensions. The assay system contained ATP, glycine, and NH_4Cl in addition to the normal constituents of a transglycosidase assay system. The reaction mixture was also buffered to a pH of 7.8 instead of 8.6. Using a reaction mixture analogous to this, Mirelman had demonstrated transpeptidation in a cell free system from *M. luteus* (*lysodeikticus*) (Mirelman *et al.*, 1972). No transpeptidase activity could be detected in either stationary or exponential-phase membrane suspensions of *M. sodonensis*. In the transpeptidase assay, transglycosidase activity was also reduced by about 25 percent for both membrane types when compared to a standard transglycosidase assay.

Mirelman had used this assay system with cell wall rather than membrane suspensions as a source of enzyme. In an attempt to duplicate his conditions, cell walls were prepared from *M. sodonensis* cells in the exponential-phase of growth as previously described. When 70 µl of a cell wall suspension (1 mg protein/ml) was assayed for transglycosidase activity it was found to contain 17 percent of the transglycosidase activity found in an equal volume of exponential-phase membrane suspension (7 mg protein/ml). Based on protein content the exponential-phase cell walls had slightly more transglycosidase activity than exponential-phase membranes. Once again, no transpeptidase activity (as evidenced by release of D alanine) could be detected in these preparations.

A. Characterization of Amidase Activity. To investigate the possibility that the unknown compound (Figure 22, Peak III) was the result of peptidoglycan breakdown, peptidoglycan was synthesized in vitro using stationary-phase membrane suspensions in a normal transglycosidase assay system. Incubation at 30°C for 3 hours resulted in about 5 nmoles of MurNAc-^{14}C-pentapeptide being incorporated into peptidoglycan. The entire reaction mixture was then boiled for 1 min, cooled, and 70 µl of exponential-phase membrane suspension was added. Incubation in the presence of these exponential-phase membranes for an additional 3 hours at 30°C resulted in conversion of 25 percent of the in vitro synthesized peptidoglycan to the unknown material seen previously (Figure 23). This explains the deceptively low levels of transglycosidase activity detected in exponential-phase membrane suspensions. It was established that rate of peptidoglycan synthesis was approximately equal for both exponential phase and stationary-phase membrane suspensions, but in the former case the in vitro synthesized peptidoglycan was subsequently being degraded as fast as it was synthesized.

B. Identification of the Amidase Product. The product of this peptidoglycan degrading enzyme was prepared in larger amounts for purification and analysis. A type 1 amidase assay was used, and by scaling up to 50 fold, roughly 210,000 dpm of UDP-MurNAc-^{14}C-pentapeptide was converted to product. The freely soluble product was separated from the membrane fragments by centrifugation at 48,000 xg for 45 min. The supernatant was then lyophilized and resuspended in a 2 ml volume of water for chromatography on a Sephadex G-25 column. The 5 ml fractions were monitored for radioactivity and positive fractions were pooled, lyophilized and streaked onto paper strips for chromatography in solvent A. Radioactive material with no UV-absorbing

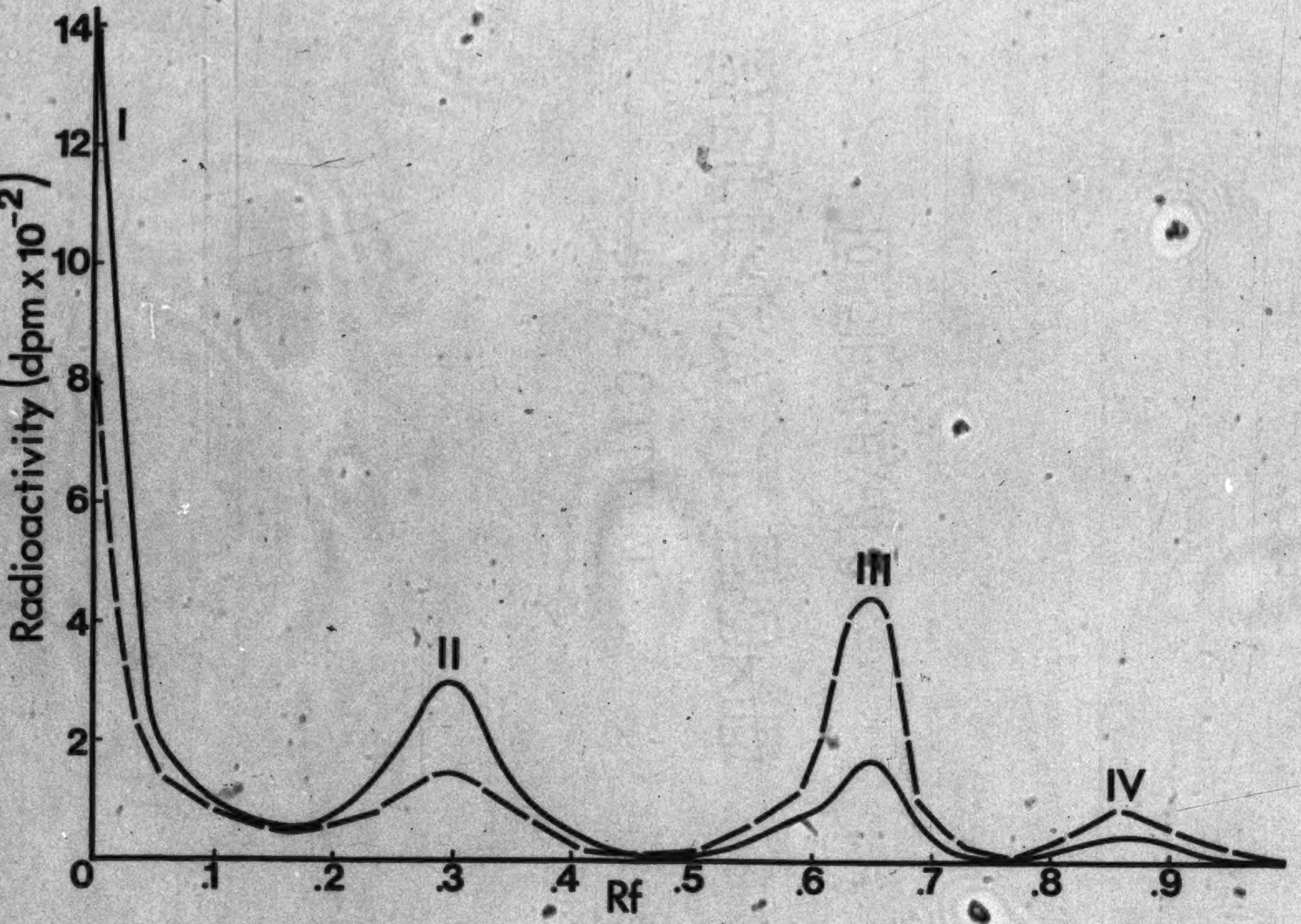
FIGURE 23

ACTION OF AMIDASE-CONTAINING EXPONENTIAL-PHASE MEMBRANE SUSPENSIONS ON *IN VITRO* SYNTHESIZED PEPTIDOGLYCAN OF *M. SODONENSIS*

Peptidoglycan was synthesized *in vitro* by stationary-phase membrane suspensions of *M. sodonensis* and then exposed to the amidase activity contained within exponential-phase membrane suspensions. After amidase exposure, the complete reaction mixture was streaked on a paper strip and chromatographed in solvent A. Radioactive products were detected by cutting the strip into 1 cm sections and counting each section in a scintillation counter.

———— peptidoglycan control

— — — peptidoglycan + amidase-containing exponential-phase membrane suspension


Radioactivity (dpm x 10^{-2})
14
12
10
8
6
4
2
0
.1
.2
.3
.4
Rf
.5
.6
.7
.8
.9
I
II
III
IV

properties was detected at an Rf of 0.65. The material was eluted from the papers and rechromatographed in solvent B. The active material, Rf of 0.32, was again eluted and desalted. The final yield of purified product was 157,000 dpm. This material was resuspended in 1 ml of water which would give a solution of 225 nmoles of peptide-containing product per ml, assuming that no dilution of the specific activity of the nucleotide substrate had taken place. Chemical assays for soluble N-acetylhexosamines, and total amino acids were performed as previously described. The results showed the product to be free ^{14}C-pentapeptide (Table IX). The peptide product contained no detectable N-acetylhexosamine. Glutamic acid and lysine were present in approximately equimolar amounts at 322 and 310 nmoles respectively. Taking their average, 316 nmoles, to be the yield of ^{14}C-pentapeptide, the specific activity of the peptide product was calculated to be 500 dpm/nmole. Alanine was present in the peptide product but at a molar ratio of 2.43 compared to 3.2 in the original substrate. The ^{14}C-pentapeptide product also differed from the original nucleotide substrate in that it contained glycine at a molar ratio of 0.30. The degradative enzyme activity was identified as an N-acetylmuramyl-L alanine amidase.

C. Substrate Specificity. The ability of the exponential-phase membrane amidase to attack substrates other than peptidoglycan was investigated. The amidase will readily degrade peptidoglycan synthesized in vitro in a transglycosidase assay system employing exponential-phase membrane suspensions, as was seen in Figure 22. However if UDP-GlcNAc was omitted from such a system so that peptidoglycan biosynthesis could not occur, there was also no evidence of amidase activity. UDP-MurNAc-^{14}C-pentapeptide

TABLE IX

COMPOSITION OF THE PRODUCT OF AMIDASE ACTION ON *IN VITRO* SYNTHESIZED PEPTIDOGLYCAN

Component	Amount[a]	Molar Ratio
Soluble N-acetylhexosamine	0	-
Glutamic acid	322	1.00
Glycine[b]	90	0.30
Alanine	784	2.43
Lysine	310	0.96

[a] nmoles per ml.

[b] Since *in vitro* synthesized peptidoglycan contains no glycine, contaminating *in vivo* synthesized peptidoglycan must also be undergoing attack by the amidase.

alone was unable to serve as an amidase substrate, even though the sensitive muramyl-L alanine bond was present. If purified MurNAc-^{14}C-pentapeptide, rather than the nucleotide, was used as a substrate the amidase was still unable to cleave the muramyl-L alanine bond.

Similarly, if in vitro synthesized peptidoglycan was first degraded by lysozyme to form a mixture of disaccharide-peptide and tetrasaccharide-peptide fragments, the muramyl-L alanine bonds in these fragments were no longer susceptible to amidase attack. The experiment was set up as follows. Peptidoglycan was synthesized in vitro using stationary-phase membrane suspensions in 3 separate transglycosidase assays. After boiling for 1 min to stop the reactions, 100 µg of lysozyme was added to two of the reaction mixtures. Sodium azide was added to all three reaction mixtures (0.02%) and all were incubated 20 hours at 37°C. After again boiling for 1 min to inactivate the lysozyme, 70 µl of exponential-phase membrane suspension was added to one of the lysozyme digests; 70 µl of TME buffer was added to the other lysozyme digest, and also to the control which was not exposed to lysozyme. After another 3 hour incubation at 30°C, all three reaction mixtures were streaked onto paper strips and chromatographed in solvent A. Amidase-containing exponential-phase membrane suspensions were unable to use either of the lysozyme digestion products as substrates (Figure 24). Lysozyme digestion completely degraded in vitro synthesized peptidoglycan and also caused a slight increase in the amount of free pentapeptide over that seen in a normal transglycosidase assay system. This may be due to a low level of contaminating amidase activity. The subsequent addition of exponential-phase membranes to a lysozyme digested transglycosidase system increased the amount of free pentapeptide, but

FIGURE 24

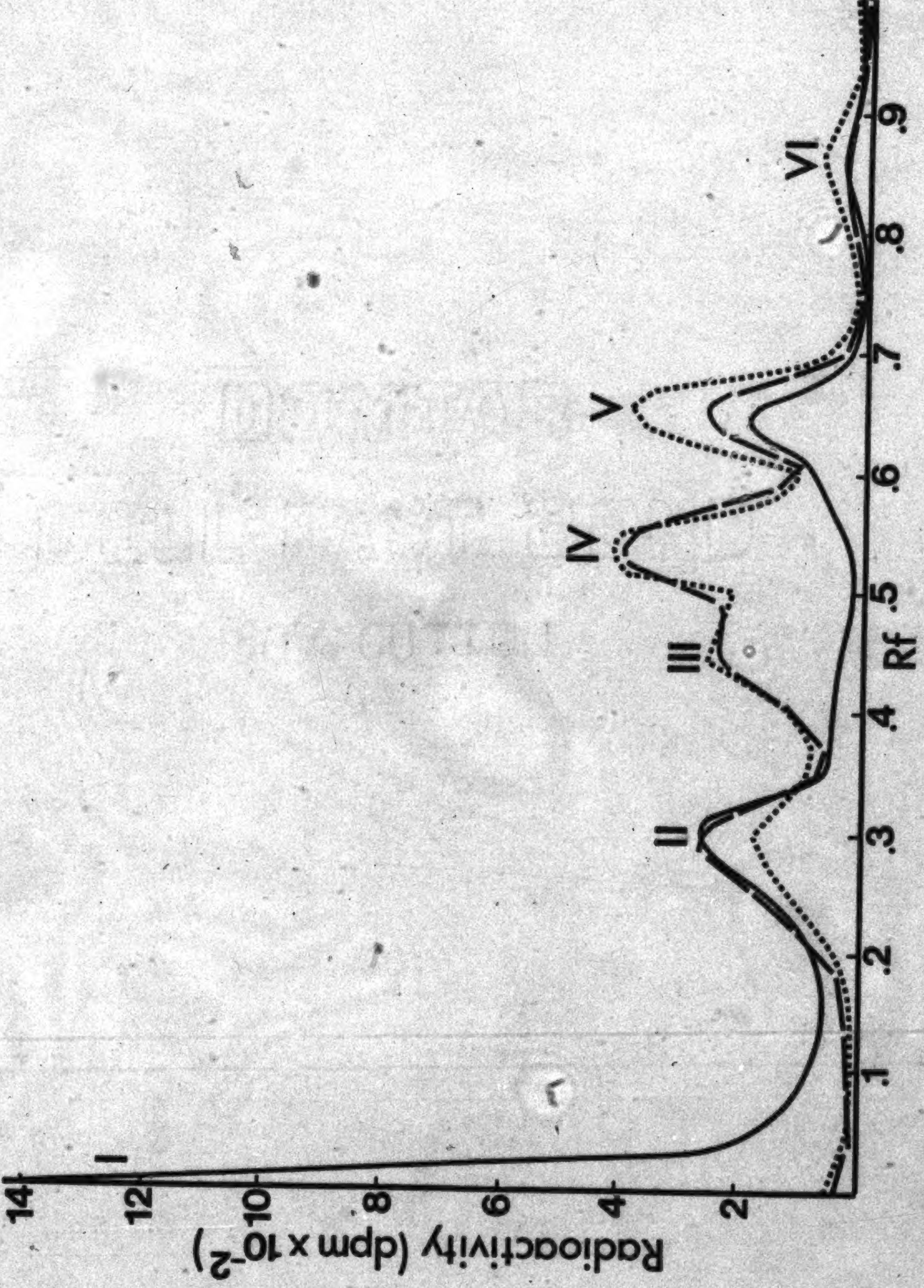
EFFECT OF LYSOZYME DIGESTION OF *IN VITRO* SYNTHESIZED PEPTIDOGLYCAN ON ITS SUSCEPTIBILITY TO AMIDASE ACTIVITY OF EXPONENTIAL-PHASE MEMBRANES OF *M. SODONENSIS*

Peptidoglycan was synthesized *in vitro* by stationary-phase membrane suspensions of *M. sodonensis* and then digested completely with lysozyme before being exposed to the amidase activity contained within exponential-phase membrane suspensions. After amidase exposure, the complete reaction mixture was streaked on a paper strip and chromatographed in solvent A. Radioactive products were detected by cutting the strip into 1 cm sections and counting each section in a scintillation counter.

——————— peptidoglycan control

— — — peptidoglycan + lysozyme

··················· peptidoglycan + lysozyme + amidase containing exponential-phase membrane suspension


Radioactivity (dpm x 10^{-2})
14
12
10
8
6
4
2
Rf
.1
.2
.3
.4
.5
.6
.7
.8
.9
I
II
III
IV
V
VI

this increase was paralleled by a decrease in the amount of free residual substrate. No change could be seen in the levels of lysozyme digested peptidoglycan products (Peaks III and IV). The residual substrate had been used by the exponential-phase membrane suspension to synthesize more peptidoglycan and the amidase digestion of this peptidoglycan caused the increase in the level of free pentapeptide.

In vitro synthesized peptidoglycan is a heterogeneous product with respect to "solubility" and precipitability by TCA. The distribution of "soluble" versus "insoluble" and TCA soluble versus TCA precipitable fractions was compared before and after exposure to amidase (Table X). In each case a type 1 amidase assay was used. After exposure to exponential-phase membranes, the "solubility" and TCA precipitability of the residual peptidoglycan was determined as described previously. Amidase digestion preferentially removes the "soluble" peptidoglycan species. Residual peptidoglycan is enriched in "insoluble" and TCA precipitable material. This is most obvious in the case of TCA precipitable material which is increased from 39 to 52 percent of the peptidoglycan upon amidase digestion.

D. Location and Function of Amidase Within the Cell. Cell wall preparations, isolated from cells in the exponential-phase of growth were separated from membranes and whole cells by differential centrifugation as described previously. The resulting cell wall suspension was an opaque yellow color. Since the yellow color of *M. sodonensis* is due to a membrane-associated carotenoid pigment while the cell wall itself is colorless, this indicated that the cell wall fragments contained attached membranous material. When the cell wall preparation was assayed for amidase activity

TABLE X

EFFECT OF AMIDASE DIGESTION ON "SOLUBILITY" AND TCA PRECIPITABILITY OF IN VITRO SYNTHESIZED PEPTIDOGLYCAN

Material Assayed	Composition of In Vitro Synthesized Peptidoglycan[a]	
	Before Amidase Digestion	After Amidase Digestion
48,000 xg pellet ("insoluble" peptidoglycan)	22.7	29
Peptidoglycan insoluble in 5% TCA	39	52

[a] percent of total peptidoglycan.

using a type 1 amidase assay it was found to be very active. Exponential-phase cell walls released 23.8 nmoles of free ^{14}C-pentapeptide per hour per mg of protein, while exponential-phase membranes released 2.0 nmoles of free ^{14}C-pentapeptide per hour per mg of protein. Even though the cell wall suspensions necessarily contained large amounts of unlabelled peptidoglycan, the amidase preferentially attacked the added in vitro synthesized peptidoglycan.

The question as to whether the amidase of M. sodonensis functions as an autolysin or in crossbridge formation was examined. Both exponential-phase and stationary-phase whole cells were harvested, washed once in ice cold TME buffer and then resuspended in TME buffer to an optical density at 600 nm of 0.5. The cell suspensions were then incubated at 30°C and changes in optical density were followed for 6 hours. Both types of cell suspension were found to be equally resistant to autolysis even though stationary-phase cells yield membranes and cell walls which are almost devoid of amidase activity while exponential-phase cells yield membranes and cell walls with high levels of amidase activity (Figure 25). This lack of correlation between presence of amidase and autolysis seems to negate such an in vivo role for the enzyme.

Even isolated exponential-phase cell walls exhibited only very slight autolytic activity (change in OD_{600} = 21 percent in 6 hours).

E. Purification of Amidase Activity.

1. Solubilization of the Amidase. Three methods of solubilization, Triton X-100 treatment, butanol extraction, and LiCl extraction (details given in Materials and Methods), were used in an attempt to release amidase activity from membrane suspensions containing 7 mg protein/ml and

FIGURE 25

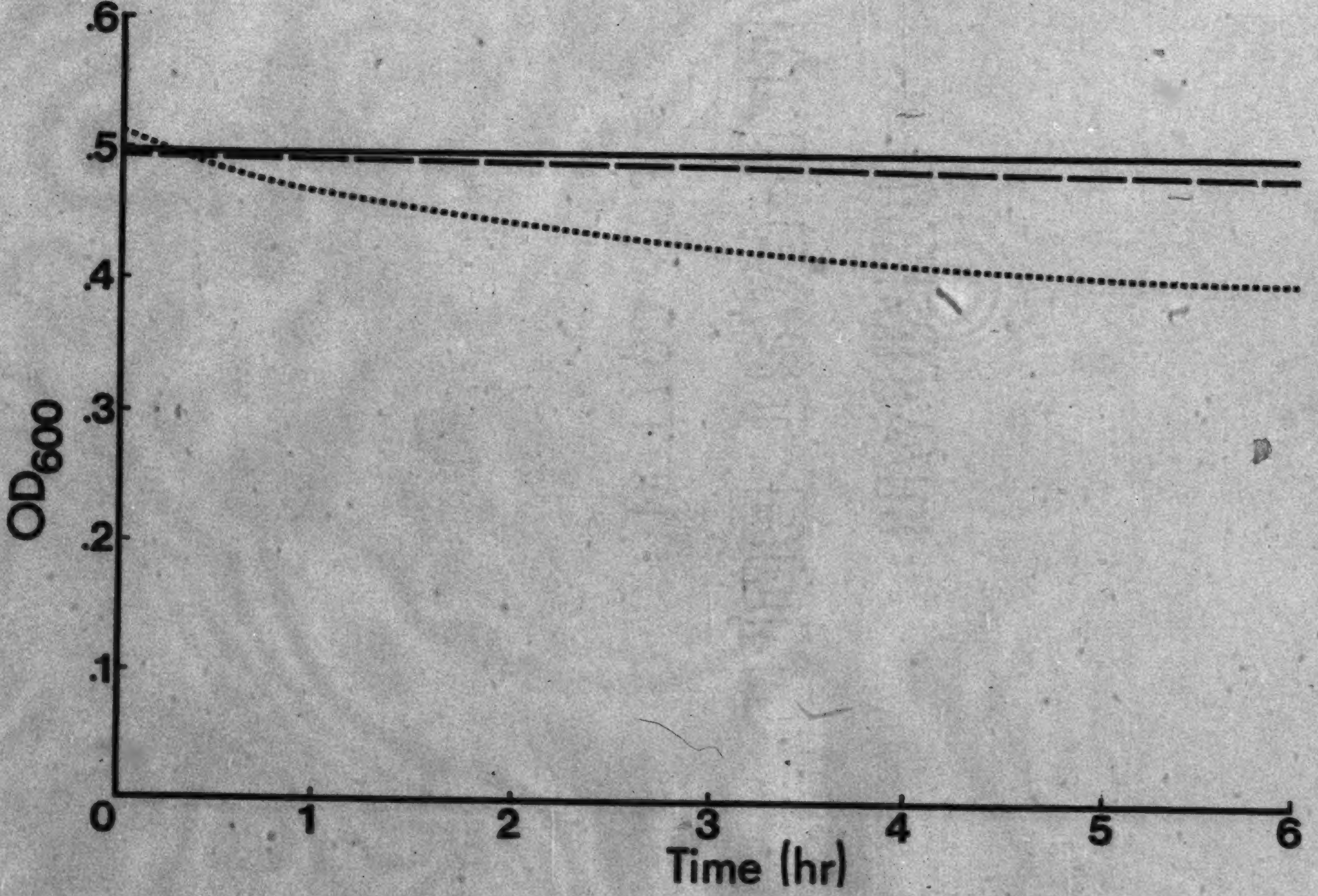
AUTOLYSIS OF EXPONENTIAL-PHASE CELLS, STATIONARY-PHASE CELLS, AND ISOLATED EXPONENTIAL-PHASE CELL WALLS OF *M. SODONENSIS*

Exponential-phase cells, stationary-phase cells, and isolated exponential-phase cell walls were washed and resuspended in TME buffer to an optical density at 600 nm of approximately 0.5. Change in optical density of the suspensions was followed during incubation at 30°C.

——————— stationary-phase cells

— — — exponential-phase cells

·················· exponential-phase cell walls


OD600
.6
.5
.4
.3
.2
.1
0
1
2
3
4
5
6
Time (hr)

cell wall suspensions containing 1 mg protein/ml. Unlike the situation with transglycosidase and pyrophosphatase, all three methods successfully solubilized amidase activity from both membranes and cell walls with no loss of ~~activity~~ (Table XI).

a. Triton X-100 treatment solubilized 82.7 percent of the initial amidase activity of the whole membrane suspension. The dark brown residual membrane material contained 63.3 percent of the initial activity for a total recovery of 146 percent. This overall increase in activity was observed for the other two methods of solubilization as well and indicated that the forces which hold the amidase to the membrane or cell wall also restrict its activity. Treatment of cell walls with 1% Triton X-100 caused them to lose their yellow color as the associated membrane material was solubilized. The clear yellow supernatant contained 82 percent of the initial activity and total activity again increased upon solubilization. Triton X-100 treatment had the disadvantage that it also solubilized the highest amount of total protein. The enzyme-containing Triton extract also contained carotenoid pigments and other lipid materials. Another disadvantage of Triton X-100 was that it caused partial solubilization of the transglycosidase and pyrophosphatase activities also present in exponential-phase membranes and cell walls.

b. Butanol extraction of cell wall and membrane suspensions also caused solubilization of high levels of amidase activity with an overall increase in activity upon solubilization. Butanol extraction however solubilized less total protein than Triton X-100 treatment and carotenoid pigments were removed into the butanol layer. Other lipid material was extracted into the butanol or trapped at the butanol-water interface.

TABLE XI

SOLUBILIZATION OF AMIDASE FROM EXPONENTIAL-PHASE MEMBRANE AND CELL WALL SUSPENSIONS OF M. SODONENSIS

Solubilization Technique	Membrane Suspensions			Cell Wall Suspensions		
	Amidase[a] Solubilized	Amidase[a] Remaining Bound	Specific[b] Activity of Soluble Extract	Amidase[a] Solubilized	Amidase[a] Remaining Bound	Specific[b] Activity of Soluble Extract
Triton X-100	82.7	63.3	3.50	82.0	88.9	24.9
Butanol Extraction	56.1	76.0	4.20	83.4	88.9	39.6
LiCl Extraction	60.0	42.0	24.0	77.5	85.7	108.5

[a] percent total original amidase activity.

[b] nmoles of ^{14}C-pentapeptide produced per hour per mg of protein.

c. LiCl extraction. The most successful solubilization method used involved washing or extracting the membranes or cell walls with 6 M LiCl in 1 M Tris HCl, pH 8.0. The resulting extracts contained 60 percent and 77.5 percent respectively of the initial amidase activity, which did not represent as great a degree of solubilization as the other two methods but the extracts contained very low levels of contaminating protein when compared to the other two methods. The LiCl extracts were also functionally pure since neither transglycosidase nor pyrophosphatase activities will withstand LiCl extraction.

2. Sephadex G-200 Column Chromatography. Cell wall and membrane suspensions were prepared from 2.4 l batches of exponential-phase cells. The yield was 10.8 ml of membrane suspension and 12 ml of cell wall suspension at 7 mg protein/ml and 1 mg protein/ml respectively. Amidase activity was solubilized from each of these preparations using LiCl extraction. The dialysed extracts from each were lyophilized and resuspended in 2.5 ml of water. The concentrates were then applied separately to a Sephadex G-200 column (2.5 x 45 cm). The column was equilibrated and eluted with TME buffer at 4°C (Figure 26). Five ml fractions were collected and absorbance at 280 nm was monitored. A 100 µl sample of each positive fraction was assayed for amidase activity using a type 1 amidase assay. Disappearance of radioactivity from the origin of the chromatograms was used as a measure of amidase activity. Both membrane and wall derived amidase activities eluted in the void volume of the column indicating that the enzyme, or enzyme containing complex which was solubilized was of large molecular weight.

LiCl extraction is normally used to solubilize cell wall-bound

FIGURE 26

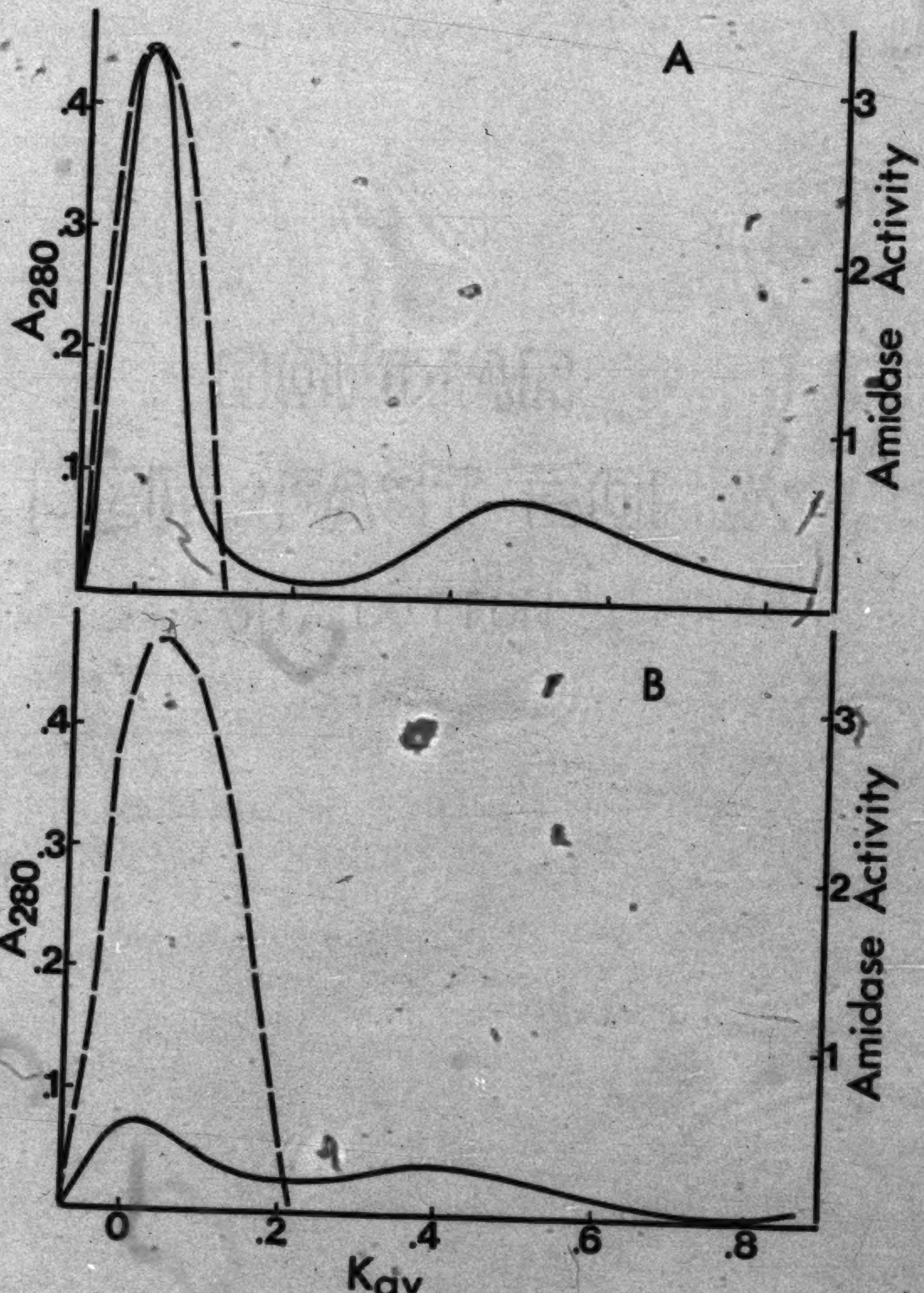
PARTIAL PURIFICATION OF SOLUBILIZED MEMBRANE AND CELL WALL DERIVED AMIDASES OF M. SODONENSIS BY GEL FILTRATION

Amidase activity was solubilized from exponential-phase membranes and cell walls by LiCl extraction as given in the text. The extracts were applied separately to a Sephadex G-200 column (2.5 x 45 cm). The column was equilibrated and eluted with TME buffer, and 5 ml fractions were collected. Absorbance at 280 nm was monitored and 100 µl amounts of positive fractions were assayed for amidase activity in a type 1 amidase assay.

A. Membrane derived amidase

B. Cell Wall derived amidase

———	absorbance at 280 nm
— — —	amidase activity expressed as nmoles of pentapeptide released per ml of enzyme


A
B
A_{280}
.1
.2
.3
.4
Amidase Activity
1
2
3
0
.2
.4
.6
.8
Kav

enzymes. In case the LiCl treatment had not extracted the typical membrane amidase, a second batch of membrane derived amidase was prepared by butanol extraction. Chromatography of this material on a Sephadex G-200 column again showed all the amidase activity eluting in the void volume. The membrane and wall amidases are similar in that their soluble forms are both of very large molecular weight.

3. Further Purification of Solubilized Amidase. DEAE cellulose column chromatography using a NaCl gradient for elution was not successful in further purifying the membrane or cell wall derived amidases. Samples of partially purified wall and membrane derived amidases, following Sephadex G-200 chromatography, were applied to a DEAE cellulose column (2.5 x 20 cm). The column was eluted with increasing concentrations of NaCl in TME buffer and fractions were assayed for amidase activity using a type 1 amidase assay. The presence of NaCl in the assay system was found to markedly affect the Rf values of substrates and products upon chromatography. Free ^{14}C-pentapeptide ran closer to the origin with increasing concentrations of NaCl, and peptidoglycan smeared away from the origin. Low concentrations of NaCl, 0.1 M, in the assay system did not appear to affect the actual production of free ^{14}C-pentapeptide, but higher NaCl concentrations, 0.5 M, caused complete inhibition of amidase activity.

Ammonium sulfate precipitation was also unsuccessful as a method of purifying the amidase activity. Ammonium sulfate was gradually added to amidase solutions at 4°C. The degree of saturation was increased stepwise, 20% at a time, and precipitated protein was collected after each increase. The precipitates were dialysed extensively against TME buffer before being assayed for amidase activity but no activity was recovered.

F. Characteristics of Solubilized Cell Wall and Cell Membrane Derived Amidases.

The amidase containing fractions from Sephadex G-200 column chromatography were used as partially purified sources of amidase for further comparison of the properties of the membrane and cell wall derived enzymes. These amidase containing fractions were pooled and concentrated to a 5 ml volume by ultrafiltration using an Amicon PM 10 filter. No amidase activity from either preparation passed through the filter. The concentrated membrane amidase contained 0.267 mg protein per ml and the cell wall amidase contained 0.056 mg of protein per ml.

1. Action of Amidase on Purified Cell Walls. The partially purified wall and membrane derived amidases were compared to the whole cell wall and membrane suspensions in their ability to degrade highly purified cell walls. The highly purified cell walls were isolated from stationary-phase cells of *M. sodonensis*. Incubation mixtures were set up containing 0.7 mg of highly purified cell walls in 70 µl of water, 0.4 ml of 0.875 M Tris HCl, pH 8.6, and 0.2 ml of 0.1 M $MgCl_2$. One ml volumes of each of four types of amidase were assayed: partially purified membrane derived amidase (0.267 mg protein/ml), partially purified wall derived amidase (0.056 mg protein/ml), exponential-phase membrane suspension (7 mg protein/ml), and exponential-phase cell wall suspension (1 mg protein/ml). For each of the four types of amidase preparation a control incubation mixture was set up in which 70 µl water replaced the highly purified cell walls. The assays and controls were incubated 3 hours at 30°C and then boiled 1 min to stop the reaction. Assays and controls were then centrifuged for 45 min at 48,000 xg, and the supernatants were desalted on a Sephadex G-15 column (1.5 x 30 cm). All fractions from the void volume to a Kav of 0.85, where the salt begins to elute, were pooled and lyophilized. The dried material

was resuspended into 200 µl of distilled water and assayed for N terminal alanine as described in Materials and Methods (Table XII).

By analysis the 0.7 mg amount of highly purified cell walls used as substrate in each incubation contained about 50 nmoles of the susceptible N-acetylmuramyl-L alanine linkage and no detectable free N terminal alanine (Johnson, 1971). The 1 ml amount of each amidase preparation used was chosen as an amount capable of releasing in excess of 50 nmoles of free ^{14}C-pentapeptide from in vitro synthesized peptidoglycan as measured in a type 1 amidase assay. The membrane derived partially purified amidase preparation was however found to have lost activity upon freezing at - 70°C. One ml of this preparation after freezing was capable of causing the release of only 25 nmoles of free ^{14}C-pentapeptide from in vitro synthesized peptidoglycan. The observed release of 53.4 nmoles of N terminal alanine by cell wall derived amidase and 24.2 nmoles by membrane derived amidase indicated that soluble amidases can attack crosslinked cell wall peptidoglycan as readily as they can attack in vitro synthesized peptidoglycan. Membrane and cell wall bound amidases caused no release of N terminal alanine above background.

2. Action of Amidase on In Vitro Synthesized Peptidoglycan Fractions. The ability of both the soluble and bound forms of exponential-phase membrane and cell wall amidases to attack fractionated, in vitro synthesized peptidoglycan was examined (Table XIII). The peptidoglycan fractions were the large and small molecular weight species of "soluble" and "insoluble" peptidoglycan which resulted from Sephadex G-200 chromatography (Figure 10, Peaks I and II). Each of the four fractions of peptidoglycan was digested with both the soluble and bound forms of cell

TABLE XII

DIGESTION OF PURIFIED CELL WALLS OF *M. SODONENSIS* BY AMIDASE

Amidase Source	N-Terminal[a] Alanine Released in Test (substrate added)	N-Terminal[a] Alanine Released in Control (no substrate added)	N-Terminal[a] Alanine Specifically Released by Added Amidase
Partially purified Membrane derived extract	83.6	59.4	24.2
Partially purified Cell wall derived extract	60.8	7.4	53.4
Exponential-phase membrane suspension	46.3	46.9	0
Exponential-phase cell wall suspension	111.6	109.7	1.9

[a] nmoles

TABLE XIII

DIGESTION OF FRACTIONATED IN VITRO SYNTHESIZED PEPTIDOGLYCAN BY AMIDASE

Substrate	Relative Amidase Activity[a]			
	Partially purified Membrane derived Extract	Exponential-phase Membrane Suspension	Partially purified Cell Wall derived Extract	Exponential-phase Cell Wall Suspension
"Soluble" large molecular weight peptidoglycan	100[a]	100[a]	100[a]	100[a]
"Soluble" small molecular weight peptidoglycan	81	71	67	30
"Insoluble" large molecular weight peptidoglycan	51	83	80	75
"Insoluble" small molecular weight peptidoglycan	87	82	87	59

[a] Amidase activity against "soluble" large molecular weight peptidoglycan was arbitrarily assigned a value of 100 and activity against the other three peptidoglycan species was expressed relative to that.

wall and cell membrane amidase using a type 2 amidase assay. Amounts of peptidoglycan which contained 900 dpm each of radioactivity were used for each assay. The peptidoglycan samples were boiled for 1 min before use. In a trial run, the amounts of exponential-phase membrane suspension, exponential-phase cell wall suspension, membrane derived amidase, and cell wall derived amidase required to give partial degradation (50-60 percent) of "soluble" large molecular weight peptidoglycan was determined. Fifty µl of membrane suspension (3.5 mg protein/ml), 50 µl of cell wall suspension (0.04 mg protein/ml), 50 µl of membrane derived amidase (0.089 mg protein/ml), and 50 µl of cell wall derived amidase (0.007 mg protein/ml) were found to be the appropriate amounts and dilutions required. These amounts were then tested for their ability to degrade the other three types of peptidoglycan fractions. The amount of radioactivity removed from the origin of the chromatograms relative to a control was used as a measure of amidase activity. The amidase activity against "soluble" large molecular weight peptidoglycan was arbitrarily assigned a value of 100 and activity against the other three peptidoglycan species was related to that value.

Membrane and cell wall forms of amidase, both soluble and bound, differ in their ability to attack these different peptidoglycan species. The "soluble", large molecular weight peptidoglycan was most readily digested by all amidase types. The cell wall suspension was not very successful at digesting small molecular weight species of peptidoglycan. In particular the "soluble" small molecular weight peptidoglycan was digested only 30 percent as well as the "soluble" large molecular weight peptidoglycan. This is significant since "soluble" small molecular weight material makes up the bulk of in vitro synthesized peptidoglycan. The solubilized form of cell wall amidase was also less well able to attack this small molecular weight material than was the solubilized form of membrane amidase. Membrane amidases were generally much more capable of digesting small molecular weight peptidoglycan.

Samples of peptidoglycan containing 900 dpm of radioactivity were used in this experiment. The hexosamine content of these peptidoglycan fractions suggested that these samples contained very different amounts of peptidoglycan because of the contribution of unlabelled peptidoglycan. The amidase digestion studies did not show such a wide variation in ability to degrade the various species. This suggests that the amidases are attacking the labelled in vitro synthesized peptidoglycan which is present in equal amounts in preference to the unlabelled pre-existing peptidoglycan. Even though the solubilized membrane and wall derived amidases can attack in vivo synthesized crossbridged peptidoglycan they appear to prefer the in vitro synthesized uncrosslinked material.

DISCUSSION

UDP-MurNAc-^{14}C-pentapeptide, accumulated by *M. sodonensis* under conditions of cation deprivation, was found to be typical of that accumulated by other related Gram-positive cocci including *M. lysodeikticus* (Bordet and Perkins, 1970). The nucleotide precursor was UV-absorbing due to the presence of UDP and contained N-acetylhexosamine, glutamic acid, lysine, and alanine in a molar ratio of 1.0: 0.99: 1.04: 3.2. Glycine, a normal substituent on the α carboxyl groups of glutamic acid residues in the native cell wall peptidoglycan of *M. sodonensis* was not present in the precursor. It appears that for this as for analogous systems, glycine is not a constituent of the nucleotide precursor accumulated intracellularly, but rather it is added to the substituent peptide while the latter is attached to the carrier lipid. UDP-MurNAc-pentapeptide can be induced to accumulate in *S. aureus* by adding antibiotics which inhibit peptidoglycan formation to the growth medium. *M. sodonensis* would not accumulate UDP-MurNAc-pentapeptide or related precursors when exposed to penicillin, cephaloridine, or cycloserine at growth inhibitory concentrations. This failure to accumulate UDP-MurNAc-pentapeptide has also been noted in other organisms such as *E. coli* and *Bacillus cereus*. UDP-MurNAc-pentapeptide is the last intracellular precursor of peptidoglycan and is suggested to be involved in control of the biosynthetic pathway. The inability of *E. coli* and *B. cereus* to accumulate this precursor has been postulated to be due to feedback inhibition by UDP-MurNAc-pentapeptide on UDP-GlcNAc-enol pyruvyl-transferase and has been demonstrated *in vitro* (Venkateswaran *et al.*, 1973). *S. aureus* would presumably lack such a control mechanism. UDP-MurNAc-pentapeptide can however be induced to accumulate in a wide range of organisms, including E. coli, B. cereus, and M. sodonensis by the addition of a chelating agent such as EDTA to the growth medium (Garrett, 1969). The resulting deprivation of divalent cations prevents all of the membrane bound and extracellular stages of peptidoglycan biosynthesis from occurring since all require Mg^{2+} as a cofactor. If feedback inhibition is the mechanism responsible for preventing the accumulation of UDP-MurNAc-pentapeptide upon exposure to antibiotics, then it should also prevent accumulation due to cation deprivation. A further weakness in this theory is that B. cereus shows a variable response to antibiotic exposure, accumulating UDP-MurNAc-pentapeptide when exposed to vancomycin but not to penicillin. Some additional mechanism for controlling the accumulation of UDP-MurNAc-pentapeptide must exist.

The UDP-MurNAc-^{14}C-pentapeptide produced by M. sodonensis was found to be a substrate for transglycosidation in an in vitro system when the other necessary substrates and cofactors were present. Glycine incorporation was not necessary for transglycosidation in vitro. In the absence of added glycine the peptidoglycan produced had free α carboxyl groups on the glutamic acid residues.

The methods of cell breakage used during preparation of cell membrane suspensions had an important effect on the resulting transglycosidase activity. Integrity of the membrane was essential for transglycosidase activity, as evidenced by the observation that the more harsh the method of cell disruption, the less transglycosidase activity which survived. Membrane yield however was reduced in the gentler methods of cell disruption due to incomplete cell breakage. Membrane suspensions produced by grinding cells with plastic beads for short periods had high levels of transglycosidase activity indicating a fairly intact membrane structure. These membrane suspensions appeared free from whole cells and cell walls upon electron microscopic examination. While no gross contamination with cell walls could be detected, the _in vitro_ peptidoglycan synthesized using these membrane suspensions was contaminated by unlabelled cell wall material as evidenced by its high hexosamine content and low specific activity. This unlabelled cell wall material must have been introduced with the membrane suspension since all other components of the transglycosidase assay system are defined. In order to withstand the buffer washes to which membrane fragments were exposed, the unlabelled cell wall material must have been attached to the membrane fragments. Some of it may represent peptidoglycan that was being synthesized at the time of cell breakage.

Membranes prepared from cells broken by partial lysozyme digestion and passage through a French pressure cell lacked transglycosidase activity. Since these membrane suspensions could still synthesize small amounts of lipid intermediates and demonstrated low levels of pyrophosphatase activity it appeared that the individual enzymes of the transglycosidase system were still active but that the overall organization necessary to bring about transglycosidation was lacking.

Succinic dehydrogenase and NADH dehydrogenase activities are membrane bound enzymes that are much less sensitive to cell breakage methods. The spatial arrangement of these enzymes must be less critical since membranes completely devoid of transglycosidase activity, destroyed mechanically, still had high levels of both of these activities.

Transglycosidase activity, detected in the cell free system from _M. sodonensis_ was very similar to the transglycosidase activity described for such systems from other organisms. The major transglycosidase product, peptidoglycan, was immobile in both solvent systems used but did show some tendency to smear in solvent A. In this solvent, peptidoglycan was present in greatest amount at the origin but significant amounts, 5-10 percent, spread as far as 4 cm away from the origin. Spreading is a characteristic of uncrosslinked peptidoglycan caused by the increased solubility of the material. _In vitro_ systems from _E. coli_ capable of synthesizing crosslinked peptidoglycan normally produce a very condensed product which will not move from the origin of the chromatogram. When penicillin is added to the reaction mixture to prevent crosslinking, a spreading product is formed which smears away from the origin (Izaki _et al._, 1968).

Other criteria commonly used to measure transpeptidation also indicated that the peptidoglycan produced by _M. sodonensis_ in the _in vitro_ assay was uncrosslinked. Free alanine was not produced during the course of peptidoglycan synthesis and the addition of cephaloridine to the assay system had no effect on the peptidoglycan product. Cephaloridine is a β lactam antibiotic and like penicillin it is presumed to function by preventing crossbridging. Since the alanine residues of UDP-MurNAc-^{14}C-pentapeptide are radioactively labelled, crossbridging, if it occurs, should cause a release of free labelled alanine. The inhibition of crossbridging by cephaloridine should increase the amount of radioactivity remaining in the peptidoglycan product by preventing the loss of the C terminal D alanine residue. Cephaloridine was used in place of penicillin because of the instability of penicillin to Tris buffers (Florey _et al._, 1949; Barnett, 1973).

Crossbridging has typically been very difficult to demonstrate _in vitro_ for organisms with complex peptidoglycan structures involving bridge peptides. Transpeptidation must be even more sensitive than transglycosidation to disruption of the native spatial arrangement of enzymes and substrates. Cell breakage by any technique causes most of the cell membrane fragments to become separated from the overlying cell wall material. It is at this interface that transpeptidation is presumed to take place so the sensitivity to cell disruption is understandable. Electron micrographs show that membrane fragments do not retain their free edges but tend to form vesicles and pull away from the cell wall. An _in vitro_ peptidoglycan synthesizing system has been developed using toluene-treated whole cells of _B. megaterium_. Since this procedure does not require mechanical methods of cell breakage, it minimizes spatial reorganization of the membrane with respect to wall and allows transpeptidation to take place (Schrader and Fan, 1974).

Minor products of the transglycosidase assay system included carrier lipid bound intermediates as well as small amounts of degraded substrate and peptidoglycan. The lipid intermediates which contained about 5 percent of the total radioactivity are commonly seen in such systems, but degradation products which have arisen enzymatically are much less common. These degradation products are due to the low levels of pyrophosphatase and amidase activity which are expressed in a transglycosidase assay system using stationary-phase membrane fragments.

Transglycosidase activity has a high pH optimum which is characteristic of transglycosidase activity in other cell free systems as well. The optimum temperature for transglycosidase activity was 30°C which corresponds with the optimum growth temperature for the organism. This correspondence of optimum transglycosidase activity with optimum growth temperature has also been noted for M. lysodeikticus where activity and growth were both maximal at 37°C. In some other organisms however, optimum activity occurred below the optimum growth temperature. S. aureus, for example showed maximum transglycosidase activity at 20°C while growth was optimal at 37°C. Lipid intermediate formation in S. aureus, in vitro, has a temperature optimum of 37°C, but the optimum temperature for the total process of transglycosidation is 20°C. It is therefore the final polymerization step (joins disaccharide-peptide units together), with a temperature optimum of 20°C, which controls the overall reaction (Strominger et al., 1966).

The requirement of transglycosidase activity for Mg^{2+} was typical of other cell free systems and illustrates why UDP-MurNAc-^{14}C-pentapeptide will accumulate under conditions of cation deprivation. Sensitivity of transglycosidase activity to high levels of Mg^{2+} has also been observed in similar cell free systems from E. coli (Izaki et al., 1968). The deleterious effect of Mg^{2+} when present during membrane preparation may be related to the inhibitory effects of high levels of Mg^{2+} in the assay system, but this sensitivity to Mg^{2+} during membrane preparation does not appear to be common among other organisms. Cell wall techoic acids are postulated to function in the control of divalent cations, particularly Mg^{2+}. These polymers bind the cations and thereby regulate their concentration in the cell membrane environment (Hughes et al., 1973). M. sodonensis is unusual among Gram-positive organisms in that it contains no cell wall techoic acids. This may account for the sensitivity of its membranes to high levels of Mg^{2+}. Membrane suspensions of other species survive the presence of Mg^{2+} at 10 to 50 mM concentrations during preparation with no apparent deleterious effects, but in M. sodonensis, 50 mM Mg^{2+} will completely destroy transglycosidase activity. This irreversible inactivation may be caused or accompanied by elution of one or more components of the system from the membrane, but addition of concentrated supernatant back to inactive membranes cannot restore activity. The inhibitory effect of Mg^{2+} during membrane preparation can be simulated by washing active membranes with a Mg^{2+} containing buffer. Considerable activity is lost but not as much as when Mg^{2+} is present in all stages of membrane preparation. This suggests that the presence of Mg^{2+} at the time of cell breakage may exert some additional stress on the membrane transglycosidase system which cannot be duplicated merely by washing the membranes.

Despite its lack of crossbridging the transglycosidase product was confirmed to be peptidoglycan by the demonstration of its susceptibility to lysozyme. Exposure to lysozyme resulted in complete digestion of the peptidoglycan into two compounds, disaccharide-peptide and tetrasaccharide-peptide. The complete sensitivity of in vitro synthesized peptidoglycan as opposed to 50 percent susceptibility of native cell wall peptidoglycan demonstrates an important structural difference between the two (Johnson and Campbell, 1972). Lysozyme resistance in native cell wall peptidoglycan is related to the extent of crossbridging and also to 0 acetyl substitution of muramic acid residues. _In vitro_ synthesized peptidoglycan was not crossbridged and, since 0 acetylation is an energy requiring process, also not 0 acetylated. This could explain the increased susceptibility to lysozyme of this material. The peptidoglycan of _M. sodonensis_ has been suggested to consist of two intertwined networks, one lysozyme soluble and one lysozyme insoluble. _In vitro_ synthesized peptidoglycan could alternatively be representative only of the lysozyme soluble network.

Peptidoglycan produced in cell free systems from _E. coli_ was crossbridged but could be completely digested by lysozyme to yield two products, disaccharide-peptide and tetrasccharide-peptide. When penicillin was incorporated into the synthetic system the peptidoglycan formed was uncrossbridged and highly soluble. Lysozyme digestion of this material resulted in only one product, disaccharide-peptide. The implication was that the tetrasaccharide-peptide product arose as a result of crossbridging (Izaki _et al._, 1968). The _in vitro_ synthesized peptidoglycan of _M. sodonensis_ was similar to the peptidoglycan produced upon exposure to penicillin in _E. coli_ in that it was highly soluble and uncrossbridged. Lysozyme digestion however resulted in production of both disaccharide-peptide and tetrasaccharide peptide. In this case the production of the tetrasaccharide-peptide fragment could not be due to crossbridging since crossbridging does not take place.

Solubility of _in vitro_ synthesized peptidoglycan is indicative that the material is uncrossbridged. Insolubility, however does not necessarily imply that the peptidoglycan is crossbridged. Seventy two to 77 percent of _in vitro_ synthesized peptidoglycan from _M. sodonensis_ was soluble. The degree of solubility of this peptidoglycan was however not affected by addition of inhibitory levels of cephaloridine which indicated that the insolubility of the remaining 23 to 28 percent of the material was not due to crossbridging. Insolubility was also not due to attachment to cell wall material since butanol extraction released the insoluble peptidoglycan into soluble form. Rather, insolubility was due to non-covalent association with the membrane fragments.

Both the "soluble" and the suspended "insoluble" in vitro synthesized peptidoglycans were not homogeneous but consisted of two distinct peptidoglycan species of different sizes. Other than attachment to the membrane, peptidoglycan fragments from "insoluble" material were similar to those of the "soluble" fraction. The majority of the in vitro synthesized peptidoglycan, 66.4 percent, was a homogeneous small molecular weight glycan strand estimated to consist of about 20 disaccharide-peptide units linked together. Most of this material was "soluble" but some was membrane bound. The remaining 33.6 percent of the peptidoglycan was large molecular weight material which was almost equally distributed between "soluble" and "insoluble" phases. This material had chain lengths varying from 70 disaccharide-peptide units to greater than 150 disaccharide-peptide units.

Peptidoglycan biosynthesis in vivo results in formation of a large insoluble network of crossbridged glycan strands, predominantly of small molecular weight. Such small molecular weight material may normally be incorporated into the cell wall during in vivo biosynthesis.

Mirelman has studied peptidoglycan biosynthesis in M. luteus (lysodeikticus) using a cell wall preparation capable of carrying out both transglycosidation and transpeptidation (Mirelman et al., 1972; Mirelman and Bracha, 1974). His work has suggested that most growth of the peptidoglycan network in vivo proceeds laterally. Complete strands of linear peptidoglycan are first synthesized on the membrane by a transglycosidase. The entire strands are then released from the membrane and attached to pre-existing peptidoglycan through crossbridge formation. Newly synthesized peptidoglycan incorporated longitudinally into cell wall material by transglycosidation accounts for only 30 percent of total incorporation. The theory of lateral cell wall growth has been further substantiated by Ward. Using a cell wall preparation from B. licheniformis, he showed that newly synthesized strands of peptidoglycan were actually being incorporated into pre-existing cell walls by transpeptidation rather than simply being crossbridged to other newly synthesized peptidoglycan strands. Furthermore, incorporation of this newly synthesized peptidoglycan into the cell wall was shown to be totally dependent on transpeptidation with transglycosidation playing no detectable part (Ward and Perkins, 1974; Ward, 1974). In a separate study of peptidoglycan biosynthesis in B. magaterium, membrane suspensions were found to synthesize large molecular weight peptidoglycan molecules which were crossbridged within themselves but which were not attached to pre-existing cell wall material (Schrader et al., 1974). If toluene-treated whole cells replaced the membrane suspensions, as a source of enzyme, then peptidoglycan was synthesized and also incorporated into pre-existing cell walls by transpeptidation (Schrader and Fan, 1974).

If this theory of lateral cell wall growth is correct, then transglycosidation proceeding in the absence of transpeptidation might be expected to result in a product consisting of linear strands of peptidoglycan of a relatively small size. The characteristics of the peptidoglycan produced *in vitro* by *M. sodonensis* would tend to support this theory. The majority of the *in vitro* synthesized material was a "soluble", uniform linear glycan molecule with a molecular weight of approximately 20,000 daltons. The large molecular weight peptidoglycan synthesized *in vitro* could represent an earlier intermediate form. Transglycosidase activity may initially result in formation of long glycan strands which are subsequently clipped into short uniform pieces (20 disaccharide-peptide units) by the controlled action of a muramidase. *In vivo*, these short strands would be incorporated into the cell wall by transpeptidation. *In vitro*, where transpeptidation does not take place, the peptidoglycan strands would be released free into the medium. Alternatively, the large molecular weight peptidoglycan synthesized *in vitro* may be due to the limited (30 percent) incorporation of newly synthesized peptidoglycan into pre-existing cell wall material by transglycosidation which was observed to occur in *M. luteus*.

Measured hexosamine contents of the separated species of *in vitro* synthesized peptidoglycan showed that unlabelled peptidoglycan contaminated the preparations to a considerable extent. This material was presumably carried over in association with the membrane fragments. Unlabelled material makes up roughly one half of the "soluble" small molecular weight peptidoglycan, but more than 9/10 of the "soluble" large molecular weight peptidoglycan. The nature of the association between labelled and unlabelled material cannot easily be determined. They elute simultaneously upon gel filtration but this only assures that the labelled and unlabelled material is of the same size. Determining molecular weights of the separate peptidoglycan fragments by chromatography on a calibrated Sephadex G-200 column had the advantage, over the $NaBH_4$ technique, that purity of the peptidoglycan sample was of little importance. Non-radioactive contaminants, including unlabelled peptidoglycan, could be ignored unless they affected the distribution coefficient of the labelled peptidoglycan species. In determining the size of the peptidoglycan species, a series of dextran standards of known molecular weights was used to calibrate the Sephadex G-200 column rather than the more common protein standards. Dextrans were presumed to more closely resemble the shape of peptidoglycan molecules than proteins would and since the distribution coefficient is affected by the shape of a molecule as well as by its molecular weight, dextran standards should give a more accurate calibration curve. Size estimation by gel filtration should be accurate providing the peptidoglycan species are not attached to protein or other non-peptidoglycan material which would make them appear larger. The estimated chain lengths of 20 disaccharide-peptide units for small peptidoglycan and 70 to 150 disaccharide-peptide units for large peptidoglycan are also based on the assumption that the molecules are single linear strands rather than a number of short strands crossbridged together.

Reduction of peptidoglycan fragments with tritiated $NaBH_4$ as a means of determining chain length had the disadvantage of requiring large amounts of peptidoglycan. The longer strands of peptidoglycan, in particular, required more material for analysis since each strand had only one reducing terminal. This method was also more sensitive to the presence of contaminating material in the peptidoglycan samples, as unlabelled peptidoglycan derivatives were measured together with labelled peptidoglycan derivatives. Contaminating non-peptidoglycan hexosamine material will also interfere with the ratio of hexosamine residues per reducing group. This technique however had the advantage that it measured actual chain length rather than molecular size. The chain lengths estimated by the two methods correlated well. The large molecular weight peptidoglycan species was found to have an average chain length of 106.5 disaccharide-peptide units by the $NaBH_4$ reduction technique. This confirmed that the large size was due to long linear strands rather than several short strands joined together by crossbridging to form a large molecule.

Pyrophosphatase activity was detected in the _in_ _vitro_ peptidoglycan synthesizing system from _M_. _sodonensis_ when UDP-MurNAc-^{14}C-pentapeptide was incubated with active membrane suspensions in the absence of UDP-GlcNAc. This control, intended to show that UDP-GlcNAc was essential for peptidoglycan biosynthesis, also showed that a second activity was present which could degrade UDP-MurNAc-^{14}C-pentapeptide. This new activity was referred to as a pyrophosphatase because carrier lipid-P-P-MurNAc-^{14}C-pentapeptide rather than UDP-MurNAc-^{14}C-pentapeptide is its true substrate. UMP, an observed end product arises during the formation of the carrier lipid linked intermediate, just as it does in the transglycosidase system. A phosphodiesterase activity which degraded UDP-MurNAc-^{14}C-pentapeptide directly, would be expected to yield UDP rather than UMP as an end product. Pyrophosphatase activity is not expressed to any appreciable extent in a normal transglycosidase assay, Since the main difference between the pyrophosphatase and the transglycosidase assay systems lies in the absence of UDP-GlcNAc from the former, UDP-GlcNAc appeared to be an inhibitor of pyrophosphatase activity. A closer examination of UDP-GlcNAc inhibition however indicated that UDP-GlcNAc inhibited pyrophosphatase activity only indirectly, by permitting transglycosidation to occur. In a transglycosidase assay system the levels of lipid linked intermediates normally stay low because they are constantly being removed to form peptidoglycan. Omitting UDP-GlcNAc from the transglycosidase assay system prevents peptidoglycan synthesis, but MurNAc-^{14}C-pentapeptide can still be transferred from the nucleotide precursor to form the carrier lipid linked intermediate with the release of UMP. The pyrophosphatase appears to function as a scavenger enzyme, releasing unproductively bound MurNAc-pentapeptide when high levels of lipid linked-MurNAc-pentapeptide accumulate, as would happen during phases of the cell cycle in which cell wall growth was not taking place. Stationary-phase membrane suspensions prepared by the lysozyme-French press method could not synthesize peptidoglycan but could synthesize small amounts of carrier lipid intermediates. The detection of correspondingly low levels of pyrophosphatase activity was consistent with the proposal that the true substrate for pyrophosphatase is MurNAc-pentapeptide linked carrier lipid, an intermediate in transglycosidation.

Several similar characteristics of transglycosidase and pyrophosphatase activities, such as an unusually high pH optimum, and requirement for Mg^{2+} suggested that the two activities may be interdependent. Pyrophosphatase activity may be a normal component of the transglycosidase enzyme system which functions as a scavenger only when peptidoglycan biosynthesis is prevented. The transglycosidase enzyme system could require a pyrophosphatase activity during the final stages of peptidoglycan biosynthesis. Disaccharide-peptide subunits are linked to the carrier lipid by a pyrophosphate bond and this bond must be broken to allow attachment of the disaccharide-peptide to a growing peptidoglycan strand. Pyrophosphatase activity could again be required to free the completed linear peptidoglycan strand from the carrier lipid (Ward, 1973).

Pyrophosphatase activity does differ markedly from transglycosidase activity in its relative insensitivity to high levels of Mg^{2+} within the assay system. Since pyrophosphatase is proposed to be a component of the transglycosidase enzyme system, it follows that the Mg^{2+} sensitive reaction must come after the pyrophosphatase step. The only major reaction taking place after cleavage of the lipid intermediates is the actual polymerization step in which the disaccharide-peptide units are joined together. Similarly pyrophosphatase is much less sensitive to high temperatures than transglycosidase activity, again suggesting that it is the final polymerization step which confers the overall sensitivity of transglycosidation to high temperature. The effect of incubation temperature on cell growth closely parallels the effect of temperature on transglycosidase activity indicating that cell wall growth may be involved in regulating overall cell growth.

The inhibitory effect of UMP when added to the pyrophosphatase assay system indicated it to be the primary end product since both uridine and UDP had little or no effect. UDP-glucose had a moderately inhibitory effect but several possible explanations for this inhibition exist. UDP-glucose can replace UDP-GlcNAc in a normal transglycosidase assay and permit synthesis of an incorrect "peptidoglycan" at about 10 percent of the maximum rate (Park and Chatterjee, 1966). This low level of synthesis may have the same effect as normal peptidoglycan biosynthesis and reduce the amount of lipid intermediate available for the pyrophosphatase. A second possibility is that UDP-glucose may be a precursor of the glucose containing polysaccharides which are present in cell walls of M. sodonensis (Johnson, 1971). Synthesis of this extracellular material has been shown to require carrier lipids (Braatz and Heath, 1974). Peptidoglycan biosynthesis has been shown to share a common pool of carrier lipid molecules with the synthetic systems for other extracellular sugar-containing polymers (Baddiley, 1972; Anderson et al., 1973). UDP-glucose could be inhibiting by forming glucose-linked carrier lipid and reducing the amount of carrier lipid available for reaction with UDP-MurNAc-^{14}C-pentapeptide.

The ability of pyrophosphatase to degrade UDP-MurNAc-^{14}C-pentapeptide through formation and cleavage of lipid intermediates may also explain why M. sodonensis will not accumulate the nucleotide precursor when exposed to penicillin or cephaloridine. Neither of these antibiotics affect the membrane-associated reactions of transglycosidation so the nucleotide precursor could be degraded to UMP and MurNAc-^{14}C-pentapeptide and returned to the cell or excreted. The accumulation of nucleotide precursors under conditions of cation deprivation is consistent with this theory since pyrophosphatase activity also has an absolute requirement for Mg^{2+}.

Solubilization attempts on transglycosidase and pyrophosphatase activities were equally unsuccessful, again suggesting a possible relationship between the two activities. Successful solubilization of the transglycosidase system would necessarily involve solubilization of several enzymes and carrier lipids in a spatial arrangement which maintains activity. Triton X-100 treatment gave the most extensive membrane solubilization of the three methods used. More than 50 percent of the membrane protein was solubilized, and approximately 20 percent of the original transglycosidase and pyrophosphatase activity was recovered in the supernatant. Butanol and LiCl extraction gave a more restricted solubilization of the membrane components and no evidence of transglycosidase or pyrophosphatase activity survived.

When exponential-phase membrane suspensions were assayed for transglycosidase activity under standard assay conditions they appeared to produce significantly less peptidoglycan than stationary-phase membranes under the identical conditions. Since peptidoglycan biosynthesis occurs most rapidly during cell growth and division, exponential-phase membranes were expected to contain at least as much transglycosidase activity as stationary-phase membranes. This low level of peptidoglycan production was due to the presence of a second enzyme activity which degraded in vitro synthesized peptidoglycan. The product of this new activity of exponential-phase membranes was isolated, analysed and found to be free ^{14}C-pentapeptide, containing glutamic acid, lysine and alanine in a molar ratio of 1.0: 0.96: 2.43. The product contained no soluble N-acetylhexosamines but did contain glycine in a molar ratio of 0.3. The pentapeptide product indicated the new activity to be an N-acetylmuramyl-L alanine amidase.

The presence of glycine in the product indicated that some of the peptides had arisen due to amidase attack on in vivo synthesized unlabelled peptidoglycan, since the UDP-MurNAc-^{14}C-pentapeptide used in the in vitro synthesis of peptidoglycan did not contain any glycine and no free glycine was present in the reaction mixture. Native cell wall peptidoglycan has glycine substituted on the free α carboxyl groups of glutamic acid in a mole for mole ratio. The presence of unlabelled peptides was also apparent from the decreased specific activity of the product peptide when compared to the original substrate. The drop in the molar ratio of alanine from 3.2 to 2.43 could also be explained if the unlabelled peptides derived from native peptidoglycan had only one C terminal D alanine residue rather than the D alanyl D alanine group present in in vitro synthesized peptidoglycan. The data suggest that the amidase product consists of a mixture of two peptides. Of the total 316 nmoles of peptide, 226 nmoles were derived from in vitro synthesized peptidoglycan and had a structure of ^{14}C ala-glu-lys-^{14}C ala-^{14}C ala, while 90 nmoles were derived from unlabelled in vivo synthesized peptidoglycan and had a structure of ala-glu(-gly)-lys-ala.

Hexosamine assays of in vitro synthesized peptidoglycan fractions indicated the presence of large amounts of unlabelled peptidoglycan such that, overall, about 2/3 of the isolated peptidoglycan was not actually in vitro synthesized but came from unlabelled in vivo synthesized peptidoglycan carried over by the membrane fragments. The pentapeptide product of amidase activity was also contaminated by peptides derived from this unlabelled peptidoglycan. The composition of the pentapeptide product however indicated that less than 1/3 of the peptides came from this unlabelled in vivo synthesized peptidoglycan. The amidase apparently degrades in vitro synthesized peptidoglycan preferentially over in vivo synthesized peptidoglycan.

An amidase activity in membranes of M. sodonensis may have an important function in crossbridge formation. In this organism crossbridging peptides are made up of substituent peptides linked together "head to tail". After transglycosidation has resulted in formation of linear glycan strands, the substituent peptides are postulated to be cleaved from their muramic acid residues by an amidase. One or more transpeptidases then join the substituent peptides together to form the bridge peptide. Mirelman has recently reported evidence to suggest that the formation of D ala-L ala and D ala-L lys bonds in peptide crossbridges are both sensitive to penicillin but at quite different levels. This would tend to support the concept of two distinct transpeptidase enzymes (Mirelman and Bracha, 1974). The characteristics of the amidase activity detected in M. sodonensis seem consistent with a synthetic role in crossbridge formation. Amidase activity was maximal in a transglycosidase assay system where exponential-phase membranes served as the source of both amidase and transglycosidase enzymes and amidase degraded the peptidoglycan as it was synthesized by the transglycosidase system. When exponential-phase membrane suspensions were used as a source of amidase to degrade peptidoglycan which had been synthesized in vitro by a stationary-phase membrane transglycosidase system, less degradation occurred and less free pentapeptide was formed. This suggests two possible explanations. One is that exponential-phase membrane peptidoglycan may be more sensitive to amidase than stationary-phase membrane peptidoglycan. Alternatively, the amidase activity may be located near the site of transglycosidation on the exponential-phase membranes, and be best able to attack the in vitro synthesized peptidoglycan as it is being formed.

The substrate specificity of the amidase indicated that it could only attack peptidoglycan. UDP-MurNAc-pentapeptide could not serve as a substrate for amidase even though it contained the susceptible muramyl-L alanine linkage. MurNAc-pentapeptide was also not acceptable as a substrate which indicated that the nucleotide portion was not responsible for the inability to use UDP-MurNAc-pentapeptide. Lysozyme digestion of in vitro synthesized peptidoglycan yielded a mixture of disaccharide-peptide and tetrasaccharide-peptide fragments. These fragments contain both GlcNAc and MurNAc-pentapeptide and yet still could not be degraded by amidase. The small molecular weight species of in vitro synthesized peptidoglycan with an estimated chain length of 20 disaccharide-peptide units was however degraded. This suggested that the amidase has a specificity towards peptidoglycan chains with some minimum size, greater than 2 disaccharide-peptide units (tetrasaccharide-peptide) but smaller than 20 disaccharide-peptide units.

Examination of exponential-phase cell wall preparations indicated that they were a good source of amidase activity. Based on protein content, exponential-phase cell walls had approximately the same amount of transglycosidase activity as exponential-phase membranes but about 12 times as much amidase activity. In this case amidase activity was measured as the ability of exponential-phase cell walls to degrade added in vitro synthesized peptidoglycan. The specific degradation of this endogenous peptidoglycan even though the cell wall itself contained high amounts of peptidoglycan again suggested that the amidase prefers to degrade in vitro synthesized peptidoglycan rather than the cell wall material which surrounds it. The high degree of solubility of in vitro synthesized peptidoglycan may be responsible for its preferential degradation by making it more accessible to the amidase. This also implies that within the cell wall environment, the amidase is held in a position which makes its own cell wall peptidoglycan inaccessible. The lack of cross-bridging of the in vitro synthesized peptidoglycan may also contribute to its enhanced degradation. In any case, this selective degradation of newly synthesized peptidoglycan is in contrast to the autolytic amidase activity of *S. aureus*. That amidase, both in vivo and in vitro, preferentially degrades old cell ~~wall~~ material (Gilpin et al., 1974).

A wide range of microorganisms are known which contain membrane or cell wall bound amidases which are not directly involved in peptidoglycan biosynthesis but rather are autolysins (Chan and Glaser, 1972; Tipper, 1969; Howard and Gooder, 1974). An examination of autolysis in exponential and stationary-phase whole cells of *M. sodonensis* indicated that the organism was very resistant to autolysis in all phases of growth. The amidase activity present in exponential-phase membrane and cell wall suspensions did not manifest itself as an autolysin in vivo.

Transglycosidase activity seemed clearly to be a membrane-associated enzyme. Transglycosidase activity in cell wall preparations was related to the presence of membrane material. Amidase activity on the other hand was found in both membrane and cell wall preparations and may be located at the wall-membrane interface with a stronger tendency to associate with cell wall material. Alternatively, there may be two distinct amidase enzymes, one membrane bound and one cell wall bound.

Similarities and differences between the two forms of amidase were examined first by solubilizing the enzymes. Triton X-100 treatment and butanol extraction are most frequently used to release membrane-bound enzymes. Both techniques released the cell wall bound form of amidase as well or better than the membrane bound form. LiCl is more typically used for solubilizing a limited range of cell wall bound enzymes, most notably autolysins. This technique also solubilized both membrane and cell wall bound forms of amidase. No distinct differences between the two types of amidase as far as extent of solubilization was noted except that membrane amidases were generally less well solubilized and less total activity was recovered. Sephadex G-200 chromatography showed both forms of LiCl solubilized amidase to be very large in size, with a molecular weight in excess of 150,000 daltons. This may indicate that the amidases have not been completely solubilized, but remain active as part of a larger soluble complex. An N-acetylmuramyl-L alanine amidase isolated from Bacillus megaterium was found to have a molecular weight of 20,000. Diplococcus pneumoniae, however also produces an amidase which is made up of subunits each with a molecular weight of 20,000 to 50,000 daltons. The complete amidase has a molecular weight of 1-3 X 10^6 daltons (Chan and Glaser, 1972; Tomasz and Westphal, 1971).

Membrane derived amidase, solubilized by butanol extraction, also eluted in the excluded volume from Sephadex G-200 indicating that the similarity in size of the two forms of amidase was not just a characteristic of the LiCl method of solubilization. Similarities also existed between the two forms of amidase, both soluble and bound, in their ability to degrade purified cell walls. The soluble forms of both types of amidase were able to attack purified cell wall peptidoglycan as readily as they would attack in vitro synthesized peptidoglycan while the bound forms of both membrane and cell wall amidases were unable to attack purified cell walls. The ability of the soluble forms of amidase to degrade crossbridged cell wall peptidoglycan may represent a broadening of specificity of the amidases which takes place on solubilization, or alternatively, the soluble forms of the amidases may simply be better able to penetrate the large insoluble cell wall peptidoglycan molecules.

The two forms of amidases, both soluble and bound, did differ in their abilities to degrade the various fractions of in vitro synthesized peptidoglycan indicating a possible difference between the two amidases. The majority of in vitro synthesized peptidoglycan is small molecular weight material with "soluble" small molecular weight material in particular making up greater than 50 percent of the total peptidoglycan. This material was only poorly degraded by cell wall amidase while membrane amidase was better able to attack it. An amidase functioning in crossbridge formation would have to use this small molecular weight peptidoglycan in order to incorporate it into pre-existing cell walls.

The cell wall and membrane forms of amidase may represent a single enzyme, but the differences in their enzymatic properties must then have been conferred upon them by their association with the cell wall or membrane. The membrane form of amidase preferred to attack peptidoglycan as it was synthesized in vitro by the transglycosidase system. It was also better able to attack the small molecular weight material typical of in vitro synthesized peptidoglycan. This amidase may represent the crossbridge forming activity. Such a function would be consistent with its presence in log-phase cells only, its preference for in vitro synthesized uncrosslinked peptidoglycan, and its lack of autolytic characteristics. Since transpeptidation did not take place in the cell free system used, the release of free pentapeptides may be the result of amidase activity proceeding in the absence of transpeptidase activity. The substituent peptides, cleaved by the amidase, were found free in the medium rather than incorporated into bridge peptides. The cell wall bound form of amidase readily degraded exogenous peptidoglycan and appeared to prefer large molecular weight species of peptidoglycan. Within the cell wall the amidase is confined such that it cannot freely attack the surrounding cell wall peptidoglycan. It may represent an amidase which opens up pre-existing peptidoglycan to allow the insertion of new material.

By controlling the proportion of these two types of amidase activity, the cell may be able to control the extent of crossbridging and influence the length of the bridge peptides in its cell wall.

The examination of peptidoglycan biosynthesis as it occurs in cell free systems from M. sodonensis has proven both interesting and rewarding. The presence of two previously unreported enzymatic activities, a pyrophosphatase and a muramyl-L alanine amidase, makes this system ideal for further investigation of peptidoglycan biosynthesis. Investigation of the peptidoglycan produced in in vitro assay systems is usually restricted to an assessment of its degree of crosslinkage. This study has attempted to examine the physical characteristics of in vitro synthesized peptidoglycan in more detail in order to compare it with the in vivo synthesized peptidoglycan of purified cell walls. Such an approach has helped to elucidate the differences between the in vitro and in vivo synthetic systems. Control and regulation is one important area of peptidoglycan biosynthesis which is only beginning to be understood. Both the pyrophosphatase and amidase activities detected in this system have characteristics which indicate they may function in controlling peptidoglycan biosynthesis. The amidase activity, especially, warrants a closer examination of the factors regulating its activity in vivo, and of its disappearance from the cells as they progress into the stationary phase of growth.

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